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The Journal of Clinical Investigation

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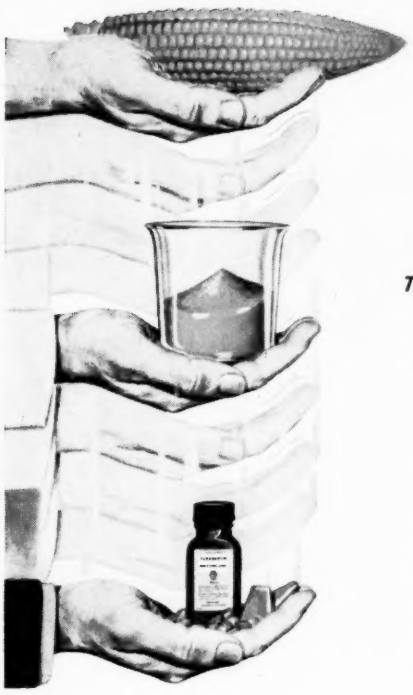

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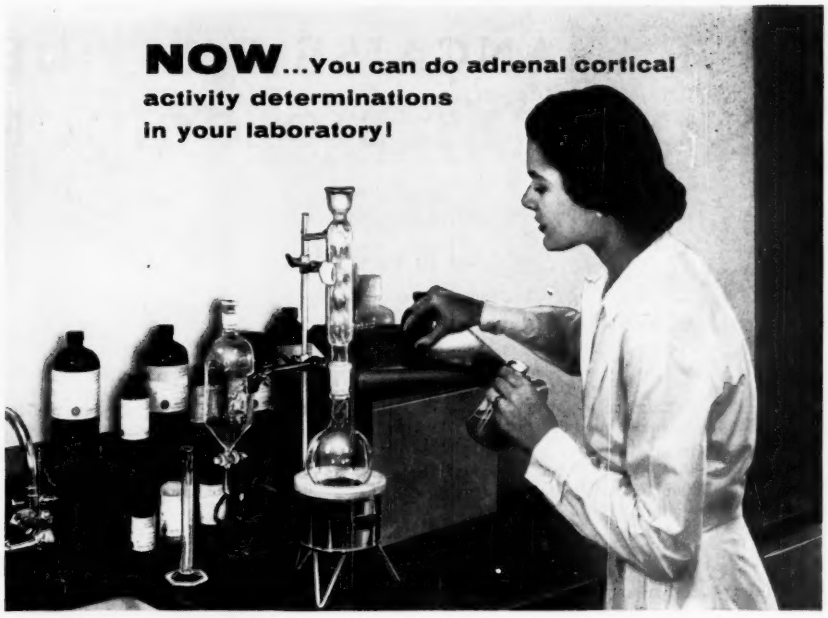

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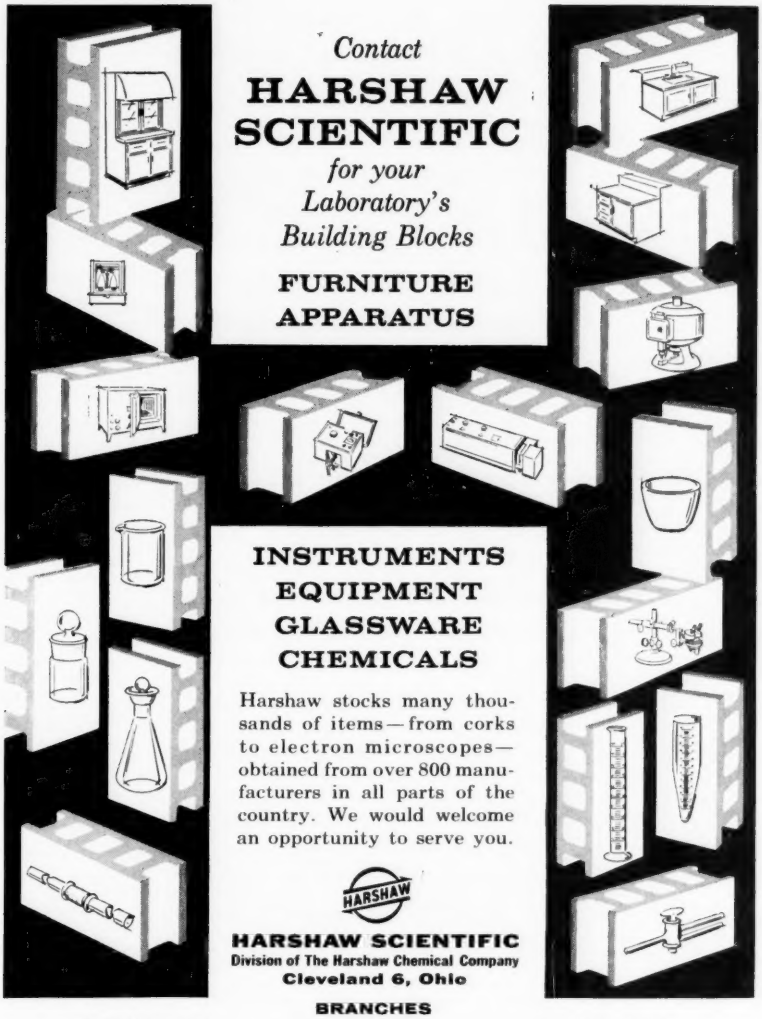

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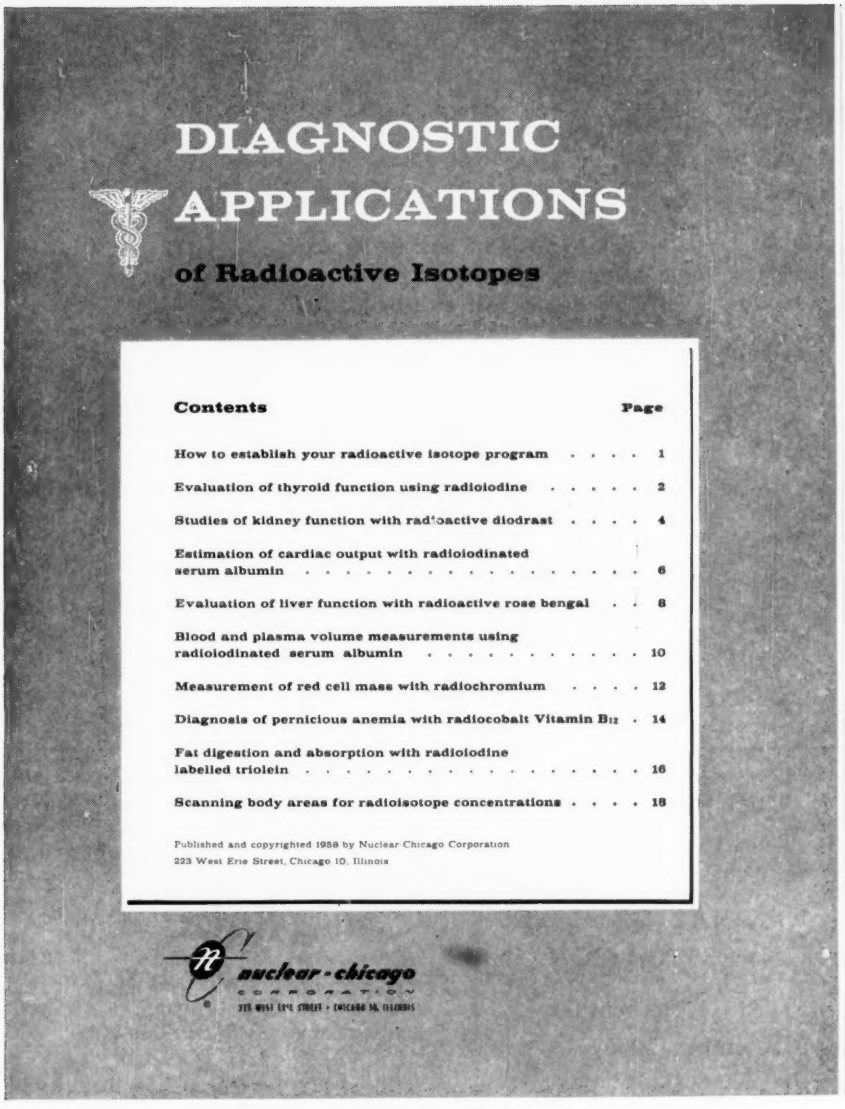
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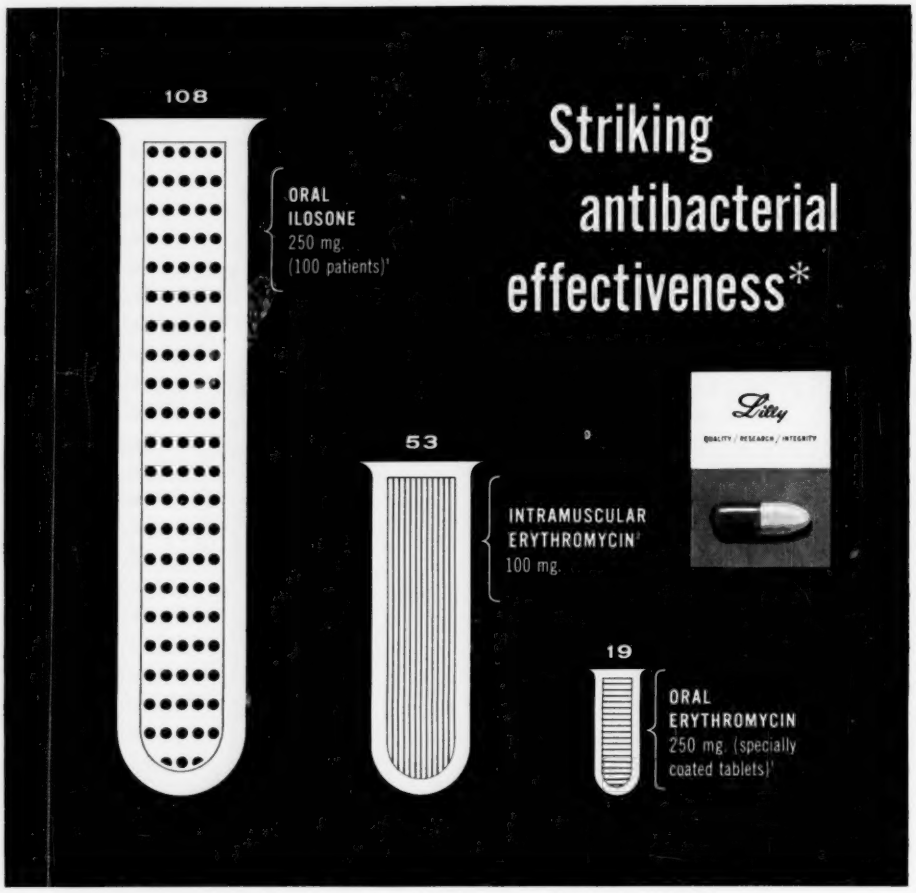

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8. Adams, R. D., and Foley, J. M. The neurological disorder associated with liver disease *in* Metabolic and Toxic Diseases of the Nervous System, H. H. Merritt and C. C. Hare, Eds. New York, Academic Press Inc., 1957, vol. 2, p. 61.

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STUDIES ON THE RENAL CONCENTRATING MECHANISM. II. EFFECT OF SMALL ACUTE CHANGES IN SOLUTE EXCRETION *

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The renal concentrating mechanism is considered to be limited by a maximal urine osmolal concentration or a maximal osmotic urine/plasma (U/P) ratio at low rates of solute excretion (1). When solute excretion is increased by the administration of large osmotic loads to hydropenic subjects urine osmolality decreases (2–4). This decrease has recently been ascribed to the limitation imposed by the existence of a maximal rate of net water reabsorption (termed $T_m{}^cH_2O$) at high rates of solute excretion and urine flow (5–7). When no osmotic load is given, solute excretion is low in hydropenic subjects so that the net rate of water reabsorption is much below its maximum. Under these circumstances the existence of a constant maximal "osmotic ceiling" independent of small changes in solute excretion and urine flow has been postulated (1, 7), but such a limit has not yet been clearly demonstrated experimentally. The present study was therefore undertaken to assess the effects of small, acute changes in solute excretion on the osmotic U/P ratio observed in hydropenic normal men. Osmotic U/P ratio was not constant but tended to vary inversely with the concurrent rate of solute excretion.

METHODS

The subjects were four paid volunteer male medical students who were selected for their ability to void small volumes of urine so that catheterization could be avoided.[1] On the day prior to the experiment subjects took no fluids after lunch and had a standard dry supper consisting of toast, meat, cheese, jelly, butter and cake. Experiments were begun at 6:00 to 7:00 a.m. the following morning. The subjects remained recumbent except to void or exercise. Urine samples were obtained at intervals of 30 minutes and blood samples approximately every two hours. The procedures described below were begun at 8:00 or 8:30 a.m. after three to four preliminary urine samples had been obtained. All experiments were performed from November to March except the urea series which was conducted in May. The following experiments were performed:

1. Resting state. Each subject remained recumbent for five to six hours.

2. Light exercise. Two subjects rode a stationary bicycle for one-half hour at 14 miles per hour against minimal resistance.

3. Venesection. In three subjects, 500 to 600 ml. of whole blood was removed in four to 20 minutes.

4. Mannitol infusion. Each subject received an infusion of 5 or 8 per cent mannitol in isotonic NaCl solution at 1 ml. per minute for two hours and at 2 ml. per minute for the next two hours.

5. Urea ingestion. Each subject ingested four doses of urea (25 Gm. per 100 ml. dissolved in orange juice) at intervals of one hour. The first two doses were 5 Gm. and the next two were 10 Gm. of urea.

Chemical methods. Creatinine was determined by the Jaffe reaction (8) using adsorption with Lloyd's reagent for the serum (9). Urea was determined by the method of Van Slyke and Cullen (10). Mannitol was determined by a chromotropic acid method (11). Sodium and potassium were determined with an internal standard flame photometer. Osmolality was measured with a Fiske freezing-point osmometer. Duplicate determinations of osmolality agreed within one per cent.

Calculations. Data on urine concentrating ability are presented in terms of the osmotic U/P ratio, and data on

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[1] Originally five subjects were selected by a "bladder emptying test." In this test the subjects voided at intervals of 30 minutes in the resting hydropenic state. The subject was considered acceptable if the creatinine clearance did not vary by more than ± 10 per cent of the mean in six periods. Subsequently, one subject showed considerable variation in creatinine clearances and his data were discarded. In 72 preliminary periods of 21 experiments on the four remaining subjects, the average deviation of the creatinine clearance from the day's mean was ± 6 per cent.

TABLE I

Summary of results of individual experiments

Experiment, subject, date	No. of periods	Range of values*						Values for regression equation†			
		V	U_{osm}	P_{osm}	Osmotic U/P ratio	C_{osm}	C_{cr}	*a*	*b*	*r*	p
		ml./min.	*mOsm./Kg.*	*mOsm./Kg.*		*ml./min.*	*ml./min.*				
Resting state											
G. C. 2/21/57	10	0.24–0.47	1,098–1,175	296–302	3.70–3.90	0.90–1.91	100–156			+0.554	>0.10
L. G. 11/24/56	10	0.51–1.07	1,038–1,223	299–304	3.40–3.99	2.09–3.60	127–145	4.47	0.308	−0.752	<0.02
R. L. 2/8/57	11	0.30–0.53	1,041–1,201	294–297	3.55–4.05	1.21–1.93	126–165	4.59	0.473	−0.732	<0.02
J. P. 11/26/56	11	0.32–0.48	1,287–1,345	301‡	4.20–4.37	1.60–2.04	131–158			+0.164	>0.10
Light exercise											
G. C. 11/17/56	8	0.34–0.82	903–1,096	288–292	3.23–3.79	1.25–2.64	102–161	4.17	0.373	−0.795	<0.02
R. L. 11/16/56	9	0.24–0.62	1,000–1,221	293–294	3.40–4.13	0.99–2.12	89–139	4.90	0.728	−0.889	<0.01
Average								4.53	0.551		
Venesection											
L. G. 12/18/56	9	0.25–0.51	1,248–1,383	297–304	4.13–4.65	1.13–2.16	120–163	5.06	0.393	−0.726	<0.05
R. L. 2/14/57	12	0.32–0.56	1,045–1,135	287–292	3.61–3.89	1.08–2.13	111–151	4.15	0.251	−0.810	<0.01
J. P. 2/12/57	10	0.28–0.59	1,214–1,313	296–303	4.05–4.40	1.67–2.30	117–153	4.92	0.361	−0.623	<0.10
Average								4.71	0.335		
Mannitol											
G. C. 12/14/56	12	0.35–1.33	880–1,097	296–302	2.94–3.63	1.26–3.95	120–156	3.94	0.236	−0.924	<0.001
L. G. 2/19/57	10	0.50–1.46	962–1,175	295–298	3.36–3.94	1.97–4.79	159–203	4.34	0.207	−0.986	<0.001
R. L. 12/15/56	11	0.29–1.31	864–1,113	290–296	2.96–3.81	1.08–3.85	109–156	4.07	0.287	−0.973	<0.001
J. P. 3/19/57	11	0.30–1.40	931–1,265	292–297	3.16–4.28	1.30–4.47	134–167	4.56	0.314	−0.956	<0.001
Average								4.23	0.261		
Urea											
G. C. 5/31/57	10	0.25–1.24	988–1,247	291–298	3.35–4.28	1.07–4.13	144–174	4.44	0.277	−0.918	<0.001
L. G. 5/30/57	9	0.35–1.27	1,075–1,359	292–303	3.56–4.65	1.63–4.88	179–225	5.09	0.304	−0.943	<0.001
R. L. 5/21/57	11	0.42–1.60	956–1,155	291–300	3.21–3.97	1.65–5.10	138–189	4.31	0.206	−0.970	<0.001
J. P. 5/29/57	11	0.39–1.71	898–1,221	296–306	2.95–4.13	1.62–5.10	132–178	4.52	0.307	−0.974	<0.001
Average								4.59	0.271		

* Abbreviations are as follows: V, urine flow; U_{osm}, urine osmolality; P_{osm}, plasma osmolality; U/P, urine/plasma; C_{osm}, osmolal clearance; and C_{cr}, creatinine clearance.

† The values given are for the constants (*a* and *b*), correlation coefficient (r) and probability (p) for the regression equation, $U/P = a - b\,C_{osm}$.

‡ No change.

solute excretion as the osmolal clearance (C_{osm}). The former expression is preferred to the use of urine osmolality alone since the data are thus corrected for variations in plasma osmolality. Osmolal clearance is calculated as: $C_{osm} = U_{osm}\ V/P_{osm}$, where U_{osm} and P_{osm} are urine and plasma osmolality and V is urine flow. This term is preferred to $U_{osm}V$ alone, not only because it corrects the data for variations in P_{osm} but also because it would measure the volume of the isotonic fluid which, according to recent evidence (12), is delivered from the distal tubules to a concentrating site in the collecting tubules.

The statistical methods used are described by Snedecor (13). The use of correlation coefficients may be questioned since the variables used contain common terms. While certain correlations might be due in whole or in part to such a common term, the negative correlation coefficients obtained in the present study do not depend on this term.

RESULTS

The results are summarized in Table I and illustrated for one subject in Figure 1. After 18 hours of water deprivation osmotic U/P ratios ranged from 3.63 to 4.65, urine flow was less than 0.5 ml. per minute and C_{osm} was 1 to 2 ml. per minute (except in Subject L.G. in whom initial values of C_{osm} were 1.6 to 3.3 ml. per minute). The U/P ratio and C_{osm} were fairly constant during the first one and one-half to two hours after waking, but subsequently a sustained increase in solute excretion was observed in the resting state in three of four experiments.

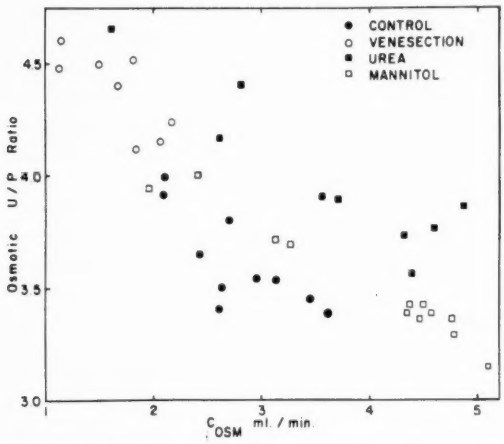

FIG. 1. OSMOTIC U/P RATIO VS. OSMOLAL CLEARANCE (C_{osm}) IN SUBJECT L. G.

Each point represents a half-hour urine collection period. The closed circles ("control") are for the resting state experiment.

After venesection, C_{osm} showed a distinct and persistent decrease in the experiment on Subject L.G., a small transient decrease in J.P., and an increase followed by a decrease in R.L., so that it was possible to observe the effects of a decreasing C_{osm}. Light exercise was followed by a sustained increase in C_{osm} in G.C. and by a transient increase in R.L. Mannitol and urea loading were used to increase C_{osm} in a predictable fashion (Figure 2). The range of C_{osm} for all data was 0.9 to 5.1 ml. per minute and the range of urine flow 0.24 to 1.71 ml. per minute. In most experiments, observations were made while C_{osm} was increasing and in this circumstance it was found that osmotic U/P ratio decreased. After venesection (and in one light exercise experiment) observations were obtained when C_{osm} was decreasing and in this circumstance osmotic U/P ratio increased. Such an inverse relationship between osmotic U/P ratio and C_{osm} was observed in each experiment except the studies on Subjects G.C. and J.P. in the resting state.[2] The linear regression equation, $U/P = a - b\ C_{osm}$, was calculated as a simple expression of this relationship. Table I gives the values of a and b, correlation coefficients (r), and probability (p) values for the linear regression equations of each experiment. The negative correlation coefficient between osmotic U/P ratio and C_{osm} was usually significant, although the constants a and b showed considerable variation. The value of the intercept a appeared chiefly to reflect variations in osmotic U/P ratio achieved at the beginning of the experiment. Variations in the slope b on the other hand are a measure of the effect of changes in solute excretion on osmotic U/P ratio. The values were between 0.206 and 0.473, except for the light exercise experiment in Subject R.L. in whom there was a more marked decrease in osmotic U/P ratio in association with a small increase in solute excretion ($b = 0.728$). This experiment may indicate that there is some impairment of the con-

[2] The lack of inverse correlation in Subject J.P. was not unexpected since changes were so small. The control data on G.C. are unexplained. The U/P ratio showed a small increase during the experiment which may have represented the effect of further dehydration, although in a previous study (14) 18 hours of water deprivation usually led to maximal osmotic U/P ratios.

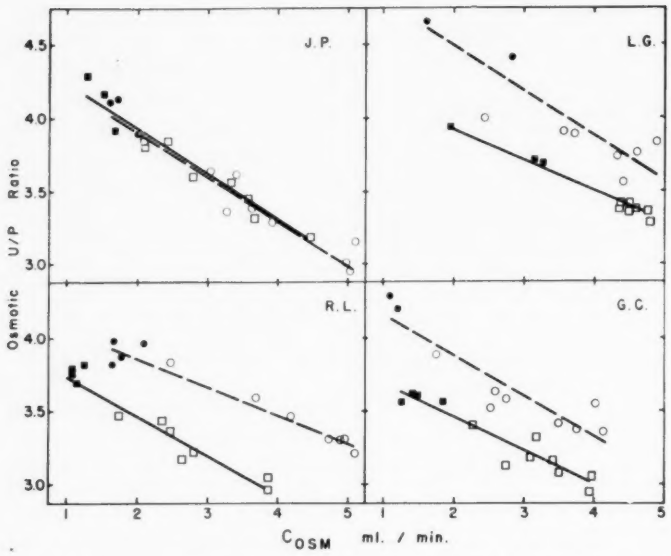

FIG. 2. OSMOTIC U/P VS. C_{osm} BEFORE AND AFTER THE ADMINISTRATION OF UREA (CIRCLES) AND MANNITOL (SQUARES)

Closed symbols represent the preliminary periods before loading. The regression lines for mannitol (solid) and urea (dashed) are drawn between the lowest and highest value for C_{osm} obtained in that experiment.

centrating mechanism with light exercise such as is seen more strikingly after heavy exercise (15). The values for the slope b were higher in some of the resting state, light excerise and venesection experiments than after mannitol and urea loading. The former experiments usually included a lower range of C_{osm} than that achieved with mannitol and urea loads and also differed in that the changes in C_{osm} were chiefly due to changes in Na and K excretion. Since the values

TABLE II

Total and partial correlations (r) and their significance (p) between osmotic urine/plasma ratio (U/P), osmolal clearance (C_{osm}), and creatinine clearance (C_{cr}) for each subject and for the massed data

Subject, age; Surface area; No. of periods	Type of correlation	U/P vs. C_{osm}		U/P vs. C_{cr}		C_{osm} vs. C_{cr}	
		r	p	r	p	r	p
G. C., 28 1.92 40	Total	−0.736	<0.001	+0.142	n.s.	+0.330	<0.05
	Partial	−0.839	<0.001	+0.604	<0.001	+0.650	<0.001
L. G., 25 1.96 38	Total	−0.785	<0.001	−0.281	n.s.	+0.696	<0.001
	Partial	−0.855	<0.001	+0.595	<0.001	+0.799	<0.001
R. L., 24 1.88 54	Total	−0.743	<0.001	−0.315	<0.05	+0.667	<0.001
	Partial	−0.753	<0.001	+0.360	<0.01	+0.680	<0.001
J. P., 26 1.90 43	Total	−0.937	<0.001	−0.614	<0.001	+0.590	<0.001
	Partial	−0.903	<0.001	−0.216	n.s.	+0.054	n.s.
All data No. periods = 175	Total	−0.635	<0.001	−0.100	n.s.	+0.625	<0.001
	Partial	−0.737	<0.001	+0.493	<0.001	+0.731	<0.001

for C_{osm} were similar in the mannitol and urea experiments, direct comparison is possible (Figure 2). The slopes of the regression line are nearly identical in the two series although osmotic U/P ratios were higher in the urea experiments in three of four subjects. In these instances, higher osmotic U/P ratios were observed in the preliminary periods before loading was begun.

There was no striking effect of variations in creatinine clearance (C_{cr}) in the present studies. There was a positive correlation between C_{cr} and C_{osm} in each subject but there was no consistent pattern of total correlation between C_{cr} and osmotic U/P ratio (Table II). When the effect of C_{osm} on osmotic U/P ratio was eliminated by determining the coefficient of partial correlation,[3] a significant positive partial correlation between C_{cr} and U/P ratio was found in three subjects. In the fourth (J.P.) the total correlation was significantly negative and the partial correlation was not significant.

Changes in plasma osmolality were small and variable, except for the consistent increase after urea loading. There was no significant correlation between the changes in plasma osmolality and the changes in osmotic U/P ratio in any group of experiments.

DISCUSSION

The acute changes in osmotic U/P ratio observed in the present study are considered to be a characteristic of the intrinsic response of the renal concentrating mechanism to small changes in solute excretion and not due to influence of extrinsic factors which might alter concentrating ability. Such extrinsic factors include a change in the production of antidiuretic hormone (ADH), variations in the prior state of hydration and variations in the prior protein intake. There is little reason to suppose that ADH production should decrease in the present experiments since dehydration was not relieved. In previous studies (14) the administration of vasopressin did not increase osmotic U/P ratio in subjects deprived of water for 18 hours; in fact, there was usually a decrease in osmotic U/P ratio after vasopressin, associated with an increase in solute excretion. Prior water loading or water deprivation (16–18) has been shown to influence the concentrating capacity despite the administration of "maximal" amounts of exogenous vasopressin. This observation has been taken to indicate that the concentrating mechanism can be influenced by the duration or degree of dehydration through the operation of some factor other than the amount of ADH immediately available. Such a factor might be directly related to the effective osmolality or inversely related to the volume of the body fluids. The present study did not test these possibilities, since most changes in osmotic U/P ratio occurred when both effective osmolality and extracellular fluid volume were nearly constant.

Although variations in protein or urea intake have been shown to alter concentrating ability (19) these changes were not observed in acute experiments. Small urea loads did not alter urine osmolality in the subjects of Epstein, Kleeman, Pursel and Hendrikx (19) who were on a low protein diet. It is possible that the decrease in U/P ratio which accompanies an increase in solute excretion was obscured by an effect of urea itself in subjects on a restricted protein intake. In the present study, U/P ratios were higher after urea loading than after mannitol loading in three of four experiments. However, this difference was associated with higher osmotic U/P ratios in the preliminary periods before the load was actually administered. Such variations in the osmotic U/P ratio after 18 hours of water deprivation have been previously observed (14) and cannot be related either to variations in solute excretion or plasma osmolality. The series of urea experiments was done in May after the other experiments of the present study had been completed so that adaptation or seasonal variation might have affected the response to 18 hours of water deprivation. With acute changes in solute excretion in an individual experiment an inverse relationship between osmotic U/P ratio and osmolal clearance was observed whether the change in solute excretion was chiefly of electrolytes, urea or mannitol. Thus the present data

[3] The coefficient of partial correlation is a convenient measure of the significance of association between two independent variables when one or more other known variables are held constant. Methods of correlation analysis rather than of multiple regression analysis were used since it could not be predetermined which of the variables was dependent.

confirm those previous studies (3, 4) which indicate that the concentrating mechanism operates independently of the contemporaneous pattern of solute reabsorption and excretion, presumably because the urine is concentrated at a site impermeable to solutes.

The decrease in osmotic U/P ratio as C_{osm} increases over the range of 1 to 5 ml. per minute has been presented as a linear regression because this expression was appropriate for most experiments. However, in some experiments (G.C., mannitol; L.G, control) the slope was somewhat steeper over a lower range of C_{osm}, suggesting a curvilinear relationship. Moreover when C_{osm} is increased progressively above 5 ml. per minute the relationship between osmotic U/P ratio and C_{osm} is clearly curvilinear (4) and U/P ratio appears to approach one as an asymptote. Attempts to extend the observations on the relationship between C_{osm} and osmotic U/P ratio to very low rates of solute excretion have thus far been unsuccessful. Such low rates can be achieved in chronic experiments by restricting dietary protein or in acute experiments after heavy exercise. Both procedures lead to lowering of the osmotic U/P ratio in hydropenic men (15, 19). This change is taken to indicate impairment of the concentrating mechanism rather than an effect of low solute excretion itself, since under these circumstances concentrating ability (as measured by net water reabsorption) is also impaired during osmotic diuresis.

The concept that the concentrating mechanism is limited by a maximum osmotic U/P ratio at low rates of solute excretion and a maximum net water reabsorption ($T_m{}^cH_2O$) at high rates of solute excretion and urine flow has been presented by Smith and co-workers (6, 7). This concept implies that there should be an appreciable range of solute excretion over which U/P ratio is constant. Boyarsky and Smith (7) reported that in their hospitalized patients (age, 24 to 74 years) who had no history of renal disease, the maximal osmotic U/P ratios after 12 to 14 hours of water deprivation were 2.2 to 3.4 and were constant up to an average urine flow of about 1 ml. per minute. There are several possible explanations for the differences between these data and the present observations. The shorter period of prior dehydration, the older age group, and possibly a poor prior protein intake may have led to the lower initial osmotic U/P ratios in Boyarsky and Smith's subjects. Moreover the conclusion that osmotic U/P ratio was constant at urine flows below 1 ml. per minute appears to be based on preliminary collection periods which show little variation in urine flow in most subjects.

The striking inverse relationship between osmotic U/P ratio and osmolal clearance obtained in the present study makes it difficult to assess the effect of other variables. While a positive partial correlation between osmotic U/P ratio and creatinine clearance was obtained in three of our subjects, the techniques used are too inaccurate to determine the effect of changes in glomerular filtration rate on concentrating ability. On the other hand, a marked decrease in glomerular filtration rate with heavy exercise (15) or constriction of the renal artery (20) is associated with definite impairment of concentrating ability.

SUMMARY

The effect of small changes in osmolal clearance on the osmotic urine/plasma ratio achieved after 18 hours of water deprivation has been studied in healthy men. Changes in osmolal clearance over the range of 0.9 to 5.1 ml. per minute and in urine flow over the range of 0.24 to 1.71 ml. per minute were observed in the resting state, after venesection, after light exercise and during mannitol and urea loading. The osmotic urine/plasma ratio was not constant. There was a significant inverse relationship between osmotic urine/plasma ratio and osmolal clearance at low rates of solute excretion and urine flow.

ACKNOWLEDGMENT

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REFERENCES

1. Smith, H. W. Principles of Renal Physiology. New York, Oxford University Press, 1956, pp. 122 ff.
2. McCance, R. A. The excretion of urea, salts, and water during periods of hydropaenia in man. J. Physiol. (Lond.) 1945, **104**, 196.

3. Hervey, G. R., McCance, R. A., and Taylor, R. G. O. Forced diuresis during hydropenia. Nature (Lond.) 1946, **157**, 338.
4. Rapoport, S., Brodsky, W. A., West, C. D., and Mackler, B. Urinary flow and excretion of solutes during osmotic diuresis in hydropenic man. Amer. J. Physiol. 1949, **156**, 433.
5. Wesson, L. G., Jr., and Anslow, W. P., Jr. Effect of osmotic and mercurial diuresis on simultaneous water diuresis. Amer. J. Physiol. 1952, **170**, 255.
6. Zak, G. A., Brun, C., and Smith, H. W. The mechanism of formation of osmotically concentrated urine during the antidiuretic state. J. clin. Invest. 1954, **33**, 1064.
7. Boyarsky, S., and Smith, H. W. Renal concentrating operation at low urine flows. J. Urol. (Baltimore) 1957, **78**, 511.
8. Bonsnes, R. W., and Taussky, H. H. On colorimetric determination of creatinine by Jaffe reaction. J. biol. Chem. 1945, **158**, 581.
9. Hare, R. S. Endogenous creatinine in serum and urine. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 148.
10. Van Slyke, D. D., and Cullen, G. E. The determination of urea by the urease method. J. biol. Chem. 1916, **24**, 117.
11. MacFadyen, D. A. Estimation of formaldehyde in biological mixtures. J. biol. Chem. 1945, **158**, 107.
12. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, Proceedings of the Eighth Symposium of the Colston Research Society, H. Heller, Ed. New York, Academic Press, 1957, pp. 157 ff.
13. Snedecor, G. W. Statistical Methods, 5th ed. Ames, Iowa, Iowa State College Press, 1956, p. 534.
14. Raisz, L. G., McNeely, W. F., and Saxon, L. G. Studies on the renal concentrating mechanism. I. Effect of vasopressin. J. Lab. clin. Med. 1958, **58**, 437.
15. Raisz, L. G., Au, W. Y. W., and Scheer, R. L. Studies on the renal concentrating mechanism. III. Effect of exercise. J. clin. Invest. 1959, **38**, 000.
16. West, C. D., Traeger, J., and Kaplan, S. A. A comparison of the relative effectiveness of hydropenia and of Pitressin® in producing a concentrated urine. J. clin. Invest. 1955, **34**, 887.
17. Jones, R. V. H., and de Wardener, H. E. Urine concentration after fluid deprivation or Pitressin Tannate in Oil. Brit. med. J. 1956, **1**, 271.
18. Epstein, F. H., Kleeman, C. R., and Hendrikx, A. The influence of bodily hydration on the renal concentrating process. J. clin. Invest. 1957, **36**, 629.
19. Epstein, F. H., Kleeman, C. R., Pursel, S., and Hendrikx, A. The effect of feeding protein and urea on the renal concentrating process. J. clin. Invest. 1957, **36**, 635.
20. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.

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STUDIES ON THE RENAL CONCENTRATING MECHANISM. III. EFFECT OF HEAVY EXERCISE

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(Submitted for publication June 6, 1958; accepted August 13, 1958)

Heavy exercise decreases urine flow, sodium excretion, glomerular filtration rate and effective renal blood flow in man (1–8). In addition, albumin, casts and red blood cells are often found in the urine sediment after exercise (9–12). Since the renal concentrating ability is a sensitive measure of renal impairment, the present study was undertaken to determine the effect of exercise on this function. Heavy exercise was found to impair concentrating ability in hydropenic normal men. This impairment was observed when the subjects exercised without prior solute loading and also during a small osmotic diuresis, but was not found when the subjects exercised during a large osmotic diuresis.

METHODS

The subjects were six paid volunteer medical students and two physicians. The medical students were selected for their ability to pass the "bladder emptying test" described in the preceding paper (13). Urine was collected by voluntary voiding in all experiments. The subjects took no fluid after lunch of the day prior to the experiment and ate a dry supper. The following experiments were performed:

I. Heavy exercise without solute loading

A. Stair-running exercise. After three to four preliminary half-hour urine collection periods four subjects (G.C., L.G., R.L. and J.P.) began running up and down three flights of stairs at 8:00 or 8:30 a.m. In 30 minutes they completed 15 trips and were exhausted. Since the subjects could not void immediately after exercise the first period was one hour long. Thereafter the subjects collected urine at half-hour intervals for three hours. In a repeated experiment, Subject R.L. received 100 mU of vasopressin intravenously just prior to exercise since an infusion could not be given during stair-running. In this experiment he was able to void at the end of exercise. In the preceding paper (13) the effect of small changes in solute excretion on the concentrating ability in the same subjects is described.

B. Step exercise. In Subjects W.A. and A.M. the exercise consisted of stepping up and down a 17 inch step every three seconds for 30 minutes. Step exercise appeared to be as exhausting as stair-running and had the advantage that it could be performed during a continuous intravenous infusion. Subject A.M. was given an infusion of 0.9 per cent saline at 0.6 ml. per minute to which vasopressin was added to provide 100 mU per hour during and after exercise. Exercise was not begun until 10:15 or 10:30 a.m. to permit comparison with studies during osmotic diuresis (see II below). Observation continued for two hours after exercise.

II. Heavy exercise during solute loading

A. Small osmotic diuresis. In three subjects (W.A., A.M., and T.T.) a diuresis was established by the infusion of 5 per cent mannitol in 0.36 per cent saline solution at a rate of 3.6 to 3.9 ml. per minute for two hours prior to exercise. Vasopressin was added to provide 200 mU per hour. Then at 10:15 or 10:30 a.m. the subjects began step exercise for one-half hour. Urine collections were obtained at intervals of 15 or 30 minutes before, during, and for 90 to 105 minutes after exercise.

B. Large osmotic diuresis. In three subjects (W.A., A.M., and L.R.) a large diuresis was established by the infusion of 5 per cent mannitol in 0.36 per cent saline at 18 to 20 ml. per minute for 90 minutes prior to exercise. Vasopressin was added to provide 200 mU per hour. Step exercise was begun at 10 or 10:30 a.m. Urine was collected at 15 minute intervals before, during, and for 105 to 120 minutes after exercise.

Chemical methods and calculations

Chemical methods and calculations are given in the preceding report (13). Glomerular filtration rate was estimated by the clearances of creatinine and mannitol.

In experiments performed without solute loading the osmotic urine/plasma (U/P) ratio was high and changes in this term could be used to evaluate concentrating ability. However, during mannitol diuresis there was a progressive fall in osmotic U/P ratio and it was increasingly difficult to evaluate changes in this variable. Under these circumstances the concentrating ability can be evaluated in terms of the relationship between net water reabsorption (T^cH_2O) and osmolal clearance (C_{osm}) (14). Such an evaluation is feasible if osmolal clearance is considered to represent the volume of isotonic fluid delivered to the concentrating site so that

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TABLE I

Effects of one-half hour of heavy exercise in four normal men *

	Before exercise	First hour	Second hour	Third hour	Fourth hour
Urine flow (V), *ml./min.*	.48 (.38–.71)	.18 (.14–.25)	.43 (.37–.50)	.60 (.55–.66)	.54 (.48–.64)
Osmolal clearance (C_{osm}), *ml./min.*	1.90 (1.51–2.76)	.53 (.42–.70)	1.30 (1.06–1.72)	1.89 (1.62–2.03)	1.91 (1.85–1.94)
Osmotic urine/plasma (U/P) ratio	3.95 (3.78–4.20)	2.90 (2.81–3.03)	3.03 (2.68–3.42)	3.18 (2.89–3.71)	3.62 (3.16–3.92)
Creatinine (C_{cr}) clearance, *ml./min.*	135 (127–143)	91 (82–102)	148 (137–159)	146 (137–161)	142 (121–155)
Na excretion ($U_{Na}V$), *μEq./min.*	85 (48–151)	23 (18–39)	48 (32–64)	63 (41–87)	88 (72–118)
K excretion (U_KV), *μEq./min.*	32 (25–43)	19 (13–25)	39 (31–56)	36 (29–44)	54 (42–71)
Plasma osmolality (P_{osm}), *mOsm./Kg.*	295 (292–297)	300 (297–302)		298 (296–300)	299 (297–300)

* Mean data and range of values are given for one and one-half to two hours before exercise and for each hour from the beginning of exercise.

T^cH_2O (calculated as C_{osm} minus urine flow) would represent the net rate of water reabsorption at that site.

RESULTS

I. Heavy exercise without solute loading

Four stair-running experiments are summarized in Table I. There was an abrupt decrease in urine flow, osmolal clearance and sodium excretion to less than half of pre-exercise values with a gradual return to pre-exercise values by the third or fourth hour. Creatinine clearance and potassium excretion decreased by about one-third with exercise and then returned to pre-exercise values within two hours. Figure 1 shows the results of a repeat stair-running experiment (Subject R.L.) and two step-exercise experiments (Subjects W.A. and A.M.) in which urine collections were obtained at the end of exercise. In these experiments the decrease in creatinine clearance as well as other functions appeared to be greater in the exercise period than in the next half-hour period. However, the likelihood of errors due to incomplete urine collection at such low urine flows is large. The osmotic U/P ratio decreased in all seven experiments. The values were 3.78 to 4.20 before exercise and decreased to 2.66 to 3.03 after exercise, except in Subject W.A. whose pre-exercise maximum osmotic U/P ratio was only 3.10 and in whom the value fell to 1.88 in the first post-exercise period. About half of the decrease in urine osmolality could be accounted for by a decrease in total electrolyte concentration (calculated as twice the sum of the change in sodium and potassium concentration), while the remainder presumably represented a decrease in urea concentration although this was not measured directly. The lowest values of U/P ratio often occurred after exercise and persisted in some instances through the fourth hour. Plasma osmolality usually increased after exercise and remained increased thereafter, the largest increment being 8 mOsm. per Kg.

II. Heavy exercise during solute loading

Exercise during osmotic diuresis also caused an abrupt decrease in urine flow, osmolal clearance and electrolyte excretion (Table II). After exercise all values rapidly returned to pre-exercise levels. With small solute loads there was a 20 to 35 per cent decrease in mannitol and creatinine clearance during exercise. With large solute loads mannitol and creatinine clearances showed no consistent change. Plasma osmolality was increased by 5 to 15 mOsm. per Kg. by solute loading but was little further altered by exercise.

TABLE II

Effect of heavy exercise during osmotic diuresis *

	Subject W. A.				Subject A. M.				Subject T. T.			
	Pre-exercise	Exercise	Post-exercise 1	Post-exercise 2	Pre-exercise	Exercise	Post-exercise 1	Post-exercise 2	Pre-exercise	Exercise	Post-exercise 1	Post-exercise 2
Small mannitol load												
V, *ml./min.*	1.73	1.11	1.73	2.18	1.48	.69	1.55	1.80	2.02†	1.22	1.86	2.47†
C_{osm}, *ml./min.*	4.00	2.60	3.64	4.75	4.41	2.01	3.88	4.96	5.73	3.38	4.81	6.27
T^cH_2O, *ml./min.*	2.27	1.49	1.91	2.57	2.93	1.32	2.33	3.16	3.71	2.16	2.95	3.80
U/P	2.31	2.34	2.11	2.18	2.98	2.93	2.52	2.76	2.84	2.76	2.59	2.54
C_{man}, *ml./min.*	69	55	66		93	50‡	76‡	86‡	122	94	115	110
C_{cr}, *ml./min.*	119	87	128	107	127	78	135	137	183	138	168	159
$U_{Na}V$, *μEq./min.*	103	58	17	99	174	26	52‡	133	271	68	157	351
U_KV, *μEq./min.*	57	35	42	50	51	29	44‡	33	107	62	61	111
	Subject W. A.				Subject A. M.				Subject L. R.			
Large mannitol load												
V, *ml./min.*	11.58	6.80	11.37	13.97	9.02	8.20	13.13	19.43	8.73†	7.86	7.50	12.93
C_{osm}, *ml./min.*	15.38	10.38	15.36	17.98	14.93	14.05	19.91	26.63	15.11	14.25	12.69	19.93
T^cH_2O, *ml./min.*	3.90	3.58	3.99	4.01	5.91	5 85	6.78	7.20	6.38	6.39	5.19	7.00
U/P	1.34	1.53	1.36	1.29	1.66	1.72	1.54	1.37	1.73	1.83	1.69	1.55
C_{man}, *ml./min.*	74	60	60	57	79	77	93	87	105	114	101	114
C_{cr}, *ml./min.*	90	90	103	120	113	108	112	92	141	156	134	160
$U_{Na}V$, *μEq./min.*	625	159	520	665	386	288	675	1,205	576	398	351	828
U_KV, *μEq./min.*	82	91	70	89	45	66	65	95	87	108	56	52

* Values represent the mean of two 15 minute urine collections except as noted. Abbreviations are the same as those used in Table I.
† One 30 minute collection.
‡ One 15 minute collection.

The effect of exercise on the concentrating ability was more difficult to evaluate during an osmotic diuresis. From previous studies (13, 15), an increase in osmotic U/P ratio would be expected when osmolal clearance decreases. Such an increase in osmotic U/P ratio was observed during exercise only in the experiments with large mannitol loads. During a small mannitol diuresis exercise was followed by a slight decrease in U/P ratio, although osmolal clearance fell markedly. This indication of impairment of concentrating ability with exercise during a small osmotic diuresis but not during a large osmotic diuresis is confirmed by examination of the relationship between osmolal clearance and water reabsorption (T^cH_2O). The data are presented graphically in Figure 2 in terms of the reciprocal values, $1/T^cH_2O$ and $1/C_{osm}$. This plot has been selected empirically because it gives a linear representation of the data and has been found to be reproducible with various types of solute loading (14). With a small osmotic load (upper part of Figure 2) this linear relationship was evident as C_{osm} was increased from 2 to 5 ml. per minute before exercise. With exercise both C_{osm} and T^cH_2O fell abruptly but T^cH_2O was lower for a given value of C_{osm}, indicating impairment of concentrating ability; this change is shown as upward displacement in the reciprocal plot. With higher solute loads there was more scatter in the plot relating $1/T^cH_2O$ and $1/C_{osm}$ but exercise did not appear to alter this relationship (lower part of Figure 2).

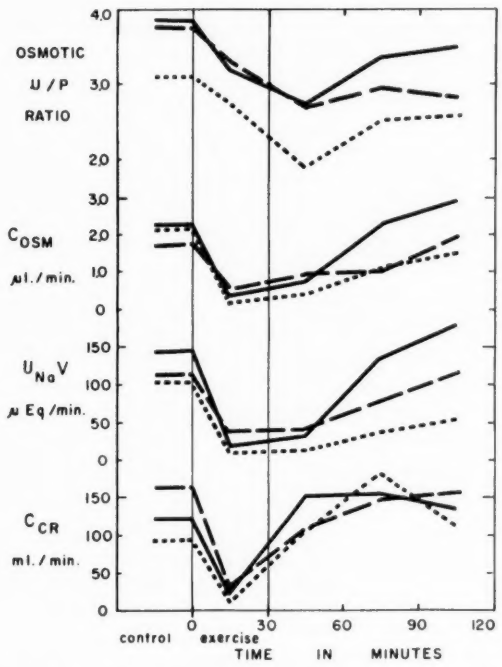

FIG. 1. THE EFFECT OF EXERCISE WITHOUT PRIOR SOLUTE LOADING ON OSMOTIC U/P RATIO, OSMOLAL CLEARANCE (C_{osm}), SODIUM EXCRETION ($U_{Na}V$) AND CREATININE CLEARANCE (C_{cr})

Data is plotted for three subjects, R.L. (solid line), A.M. (dashed line), and W.A. (dotted line).

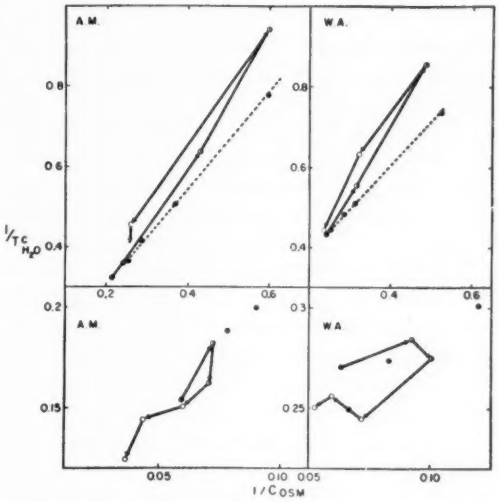

FIG. 2. THE RELATIONSHIP BETWEEN NET WATER REABSORPTION (T^cH_2O) AND OSMOLAL CLEARANCE (C_{osm}) EXPRESSED AS THE RECIPROCALS $1/T^cH_2O$ AND $1/C_{osm}$

Upper figures represent two studies during a small mannitol diuresis and lower figures two studies during a large mannitol diuresis. Each point represents a 15 minute period. Closed circles represent pre-exercise values, half closed circles values during heavy exercise, and open circles values after exercise.

DISCUSSION

The present data demonstrate that heavy exercise leads to impairment of the concentrating ability in hydropenic normal men. This impairment was observed in experiments without solute loading and also during a small osmotic

diuresis, but not during a large osmotic diuresis. The effect could not be prevented by the administration of vasopressin and is therefore considered not to be the consequence of a decrease in endogenous antidiuretic hormone production. It is difficult to ascribe the decrease in osmotic U/P ratio to the decrease in total solute excretion which occurs with heavy exercise, since in most other circumstances an inverse relationship between U/P ratio and solute excretion is observed (13). On the other hand a marked decrease in sodium excretion might be an important factor, since there is evidence that active sodium transport may be the initial process leading to the formation of a concentrated urine (16, 17). When heavy exercise was performed during a large mannitol diuresis there was an abrupt decrease in sodium excretion but no impairment of concentrating ability. However, the absolute rate of sodium excretion during exercise remained high.

Another regularly observed concomitant of the exercise effect on concentrating ability was a decrease in creatinine or mannitol clearance. This effect was not observed when exercise was conducted during a large mannitol infusion. The data, however, are subject to errors due to incomplete bladder emptying and dead space effects. Presumably, decrease in renal blood flow also occurred, since para-aminohippurate clearance has been shown to decrease progressively with increasingly severe exercise (5). Acute impairment of concentrating ability has also been observed in dogs given hexamethonium (18). In these experiments impairment persisted beyond the time when solute excretion and renal hemodynamic changes had returned to control values. Such prolonged depression of osmotic U/P ratio was also observed after exercise without solute loading although this could have been due to renal dead space effects. Recently Berliner, Levinsky, Davidson and Eden (19) have noted a reduction in concentrating ability following constriction of the renal artery in dogs.

Several mechanisms for impairment of concentrating ability are suggested by these experimental results. Perhaps the simplest mechanism would be impairment due to reduction of the supply of oxygen to the active transport system at the concentrating site. In a few experiments we have not found impairment of concentrating ability in subjects breathing 10 per cent oxygen. However, there was renal vasodilatation which may have compensated for the lower O_2 tension. If the concentrating mechanism operates by establishing a high sodium and urea concentration in the renal medulla leading to the passive reabsorption of water from the collecting tubules then the supply of these solutes will be a limiting factor in concentrating ability. This supply can be reduced both by decreased filtration and by increased proximal reabsorption. A reduced flow rate in Henle's loops might also impair the hairpin countercurrent mechanism postulated by Wirz, Hargitay and Kuhn (20–22) for concentrating the fluid in these loops. Finally the slowing of blood flow through the renal medullary capillaries could impair concentrating ability if these vessels provide the only means for returning water reabsorbed from the collecting ducts to the circulation. There is at present no evidence which would lead to the selection of one of these alternatives as the cause of impaired renal concentrating ability after exercise.

SUMMARY

Heavy exercise causes impairment of concentrating ability and a decrease in creatinine and mannitol clearance in hydropenic men who are studied without prior solute loading or during a small mannitol diuresis. During a large mannitol diuresis there is no distinct change in concentrating ability or in creatinine and mannitol clearance with exercise. In all three groups exercise causes an abrupt decrease in total solute excretion and particularly in sodium excretion.

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REFERENCES

1. Grande Covian, F., and Rehberg, P. B. Über die Nierenfunktion während schwerer Muskelarbeit. Skand. Arch. Physiol. 1936, **75**, 21.
2. Eggleston, M. G. The effect of exercise on chloride excretion in man during water diuresis and during tea diuresis. J. Physiol. (Lond.) 1943, **102**, 140.

3. Barclay, J. A., Cooke, W. T., Kenney, R. A., and Nutt, M. E. The effects of water diuresis and exercise on the volume and composition of the urine. Amer. J. Physiol. 1947, **148**, 327.
4. White, H. L., and Rolf, D. Effects of exercise and of some other influences on the renal circulation in man. Amer. J. Physiol. 1948, **152**, 505.
5. Chapman, C. B., Henschel, A., Minckler, J., Forsgren, A., and Keys, A. The effect of exercise on renal plasma flow in normal male subjects. J. clin. Invest. 1948, **27**, 639.
6. Merrill, A. J., and Cargill, W. H. The effect of exercise on the renal plasma flow and filtration rate of normal and cardiac subjects. J. clin. Invest. 1948, **27**, 272.
7. Kattus, A. A., Sinclair-Smith, B., Genest, J., and Newman, E. V. The effect of exercise on the renal mechanism of electrolyte excretion in normal subjects. Bull. Johns Hopk. Hosp. 1949, **84**, 344.
8. Bucht, H., Ek, J., Eliasch, H., Holmgren, A., Josephson, B., and Werkö, L. The effect of exercise in the recumbent position on the renal circulation and sodium excretion in normal individuals. Acta physiol. scand. 1953, **28**, 95.
9. Collier, W. Functional albuminuria in athletes. Brit. med. J. 1907, **1**, 4.
10. Barach, J. H. Physiological and pathological effects of severe exertion (the marathon race) on the circulatory and renal systems. Arch. intern. Med. 1910, **5**, 382.
11. Gardner, K. D., Jr. "Athletic pseudonephritis"—alteration of urine sediment by athletic competition. J. Amer. med. Ass. 1956, **161**, 1613.
12. Alyea, E. P., and Boone, A. W. Urinary findings resulting from nontraumatic exercise. Sth. med. J. (Bgham, Ala.) 1957, **50**, 905.
13. Raisz, L. G., and Scheer, R. L. Studies on the renal concentrating mechanism. II. Effect of small acute changes in solute excretion. J. clin. Invest. 1959, **38**, 1.
14. Raisz, L. G., Au, W. Y. W., and Scheer, R. L. The relationship between net water reabsorption and osmolal clearance as a measure of renal-concentrating activity (abstract). Clin. Res. 1958, **6**, 284.
15. Rapoport, S., Brodsky, W. A., West, C. D., and Mackler, B. Urinary flow and excretion of solutes during osmotic diuresis in hydropenic man. Amer. J. Physiol. 1949, **156**, 433.
16. Ullrich, K. J., and Jarausch, K. H. Untersuchungen zum Problem der Harnkonzentrierung und Harnverdünnung. Pflüg. Arch. ges. Physiol. 1956, **262**, 537.
17. Ullrich, K. J. Aktiver Natriumtransport und Sauerstoffverbrauch in der äusseren Markzone der Warmblüterniere. Pflüg. Arch. ges. Physiol. 1957, **266**, 55.
18. Del Greco, F., Corcoran, A. C., and Page, I. H. Hyposthenuria and other renal effects of hexamethonium in the hydropenic anesthetized dog. J. Pharmacol. exp. Ther. 1956, **117**, 434.
19. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.
20. Wirz, H., Hargitay, B., and Kuhn, W. Lokalisation des Konzentrierungsprozesses in der Niere durch direkte Kryoskopie. Helv. physiol. Pharmacol. Acta 1951, **9**, 196.
21. Wirz, H. The production of hypertonic urine by the mammalian kidney *in* Ciba Foundation Symposium on the Kidney. Boston, Little, Brown & Co., 1954, p. 38.
22. Wirz, H. The location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, Proceedings of the Eighth Symposium of the Colston Research Society, H. Heller, Ed. New York, Academic Press, 1957, pp. 157 ff.

THE DESTRUCTION OF GLUCAGON, ADRENOCORTICOTROPIN AND SOMATOTROPIN BY HUMAN BLOOD PLASMA *

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(Submitted for publication July 9, 1958; accepted August 13, 1958)

Human blood contains an inactive proteolytic enzyme, plasminogen, which may be converted *in vitro* to its active form, plasmin, either spontaneously or by the addition of various agents (1). A similar activation is believed to occur *in vivo* after exposure to a variety of noxious circumstances (2–6). Since plasmin catalyzes the hydrolysis of corticotropin A (7), it is possible that circumstances which induce the activation of plasmin will result also in an increase in the rate of destruction of adrenocorticotropic (ACTH) and other polypeptide hormones.

The present study was designed to determine the *in vitro* hydrolytic action of human blood plasma on ACTH, somatotropin, glucagon and insulin before and after the conversion of plasminogen to plasmin by streptokinase.

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METHODS

Human blood plasma prepared from freshly drawn heparinized venous blood, plasminogen prepared from fresh plasma by Milstone's procedure (8), and two different preparations of plasmin (9, 10) were employed in this study. A preparation of streptokinase (SK) which contained approximately 4,000 units per mg. was used to activate the conversion of plasminogen to plasmin. Crystalline glucagon (molecular weight [M. W.] = 4,000), a preparation of ACTH which assayed 5.3 units per mg. (assumed M. W. = 10,000), glucagon-free insulin (assumed M. W. = 12,000) and a preparation of somatotropin (assumed M. W. = 46,800) were used as substrates. Substrates labeled with I^{131} by the method described by Bauman, Rothschild, Yalow and Berson (11) were used as tracers in a mixture with unlabeled substrate.

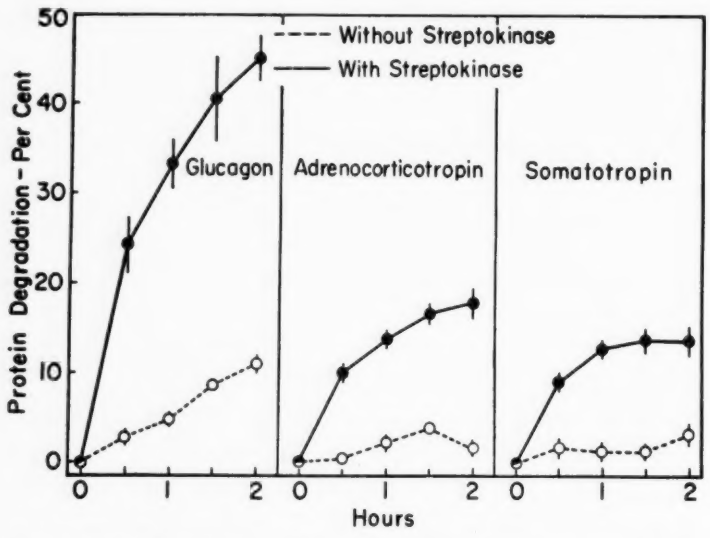

FIG. 1. THE DEGRADATION OF GLUCAGON, ADRENOCORTICOTROPIN AND SOMATOTROPIN BY NATIVE PLASMA AND BY STREPTOKINASE-ACTIVATED PLASMA

The per cent (mean plus or minus standard error) degraded at each interval was computed as indicated in text.

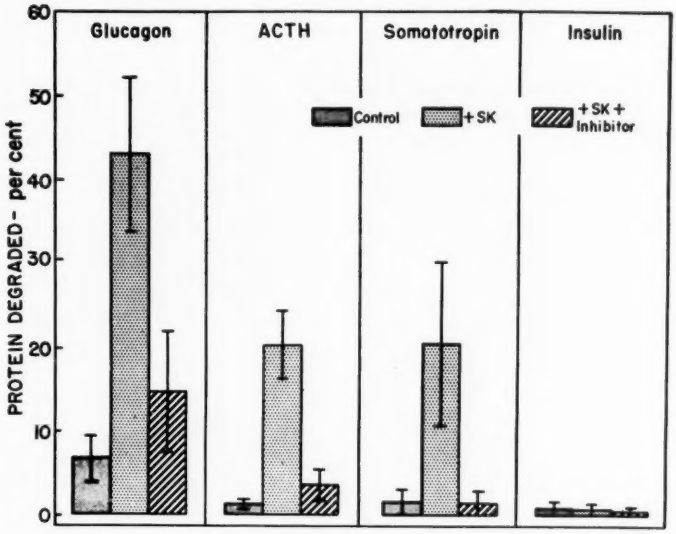

FIG. 2. THE DEGRADATION OF GLUCAGON, ADRENOCORTICOTROPIN, SOMATOTROPIN AND INSULIN BY CRUDE PLASMINOGEN, STREPTOKINASE-ACTIVATED PLASMINOGEN AND STREPTOKINASE-ACTIVATED PLASMINOGEN IN THE PRESENCE OF CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

The per cent (mean plus or minus standard error) degraded during a 90 minute period of incubation was computed as indicated in text.

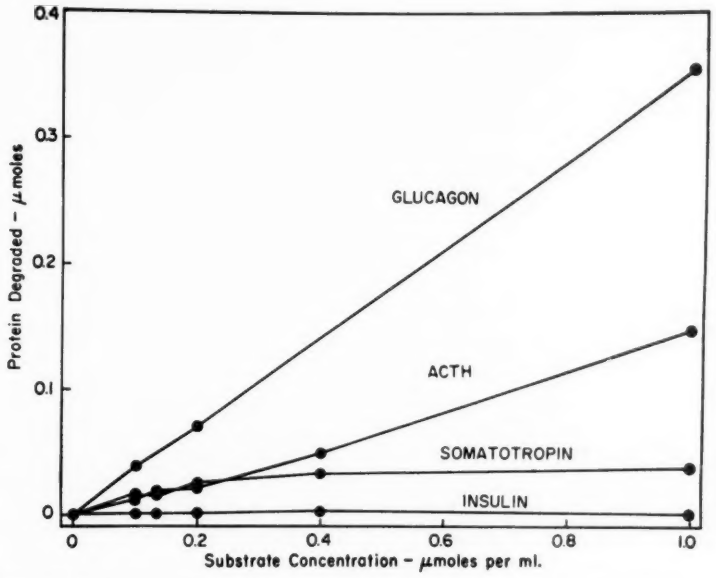

FIG. 3. DEGRADATION OF VARIOUS CONCENTRATIONS OF GLUCAGON, ADRENOCORTICOTROPIN, SOMATOTROPIN AND INSULIN BY STREPTOKINASE-ACTIVATED PLASMINOGEN

The quantity of protein degraded was computed from the TCA soluble radioactivity after incubation for 60 minutes at 37° C.

The activity of human plasma was determined by incubating 1 ml. plasma with 0.2 μM or more of the substrate containing tracer quantities of labeled substrate in 0.5 ml. McIlvaine buffer (12) at pH 7.8 and 0.5 ml. buffer containing 1,000 units streptokinase. Each substrate was incubated with an aliquot of the same sample of plasma. The mixtures were incubated in test tubes in a Dubnoff shaker at 37° C. At designated intervals the reaction was stopped by the addition of an equal volume of 10 per cent trichloracetic acid (TCA) to the appropriate tubes. One set of controls, designated to assess any preformed proteolytic activity of the plasma, contained plasma and substrate but the streptokinase was added after the TCA. A second set of controls consisted of incubation of the substrate with streptokinase and the addition of the plasma after the precipitation with TCA. The radioactivity of the whole TCA precipitated incubation mixture and of the TCA soluble portion were assayed in a well-type scintillation counter and after appropriate corrections the degradation of each substrate was computed as described previously (13).

Crude plasminogen was prepared by precipitating the euglobin fraction of fresh human blood plasma with 19 volumes of distilled water and adjusting to pH 5.2 with 0.5 per cent acetic acid. The mixture was centrifuged and the precipitate from two volumes plasma was resuspended in one volume of McIlvaine's buffer. Each incubation mixture consisted of 1 ml. of the euglobulin solution, 0.5 ml. buffer containing 0.2 μM or more of the mixture of labeled and unlabeled substrate, and 0.5 ml. McIlvaine buffer at pH 7.8. In a second set of tubes with the same mixture, the buffer contained 1,000 units streptokinase. In a third set of tubes consisting of the same volumes of plasminogen, substrate and streptokinase, the buffer also contained 2.4 mg. of five times crystallized soybean trypsin inhibitor. At designated intervals the incubation mixture was precipitated with an equal volume of 10 per cent TCA. The proportion of the substrate which became TCA soluble was determined as above.

In order to establish that the rate at which a substrate yielded TCA soluble radioactive fragments represents the rate of its hydrolysis, the same concentration of plasminogen was incubated with 1 μM of each substrate and the rate of release of TCA soluble radioactive fragments and the increase in the optical density at 280 mμ were determined on the same filtrates.

The effect of human plasmin on the degradation of the various proteins was determined by incubating 1 ml. plasmin solution (from 0.2 to 0.5 mg. in 1 ml. McIlvaine's buffer) with 1 ml. buffer containing from 0.1 to 1.0 μM of the mixture of labeled and unlabeled substrate.

RESULTS

Incubation of fresh native plasma (final concentration, 0.5 ml. per ml. incubation mixture)

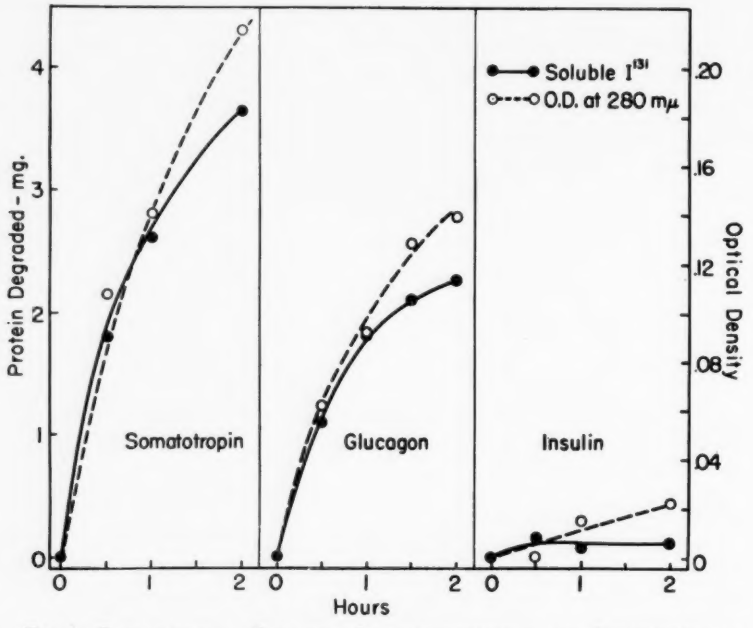

FIG. 4. DEGRADATION OF GLUCAGON, ADRENOCORTICOTROPIN AND SOMATOTROPIN BY STREPTOKINASE-ACTIVATED CRUDE PLASMINOGEN AS MEASURED BY THE INCREASE IN TCA SOLUBLE RADIOACTIVE FRAGMENTS AND THE OPTICAL DENSITY AT 280 Mμ

The quantity of protein degraded was computed as indicated in text.

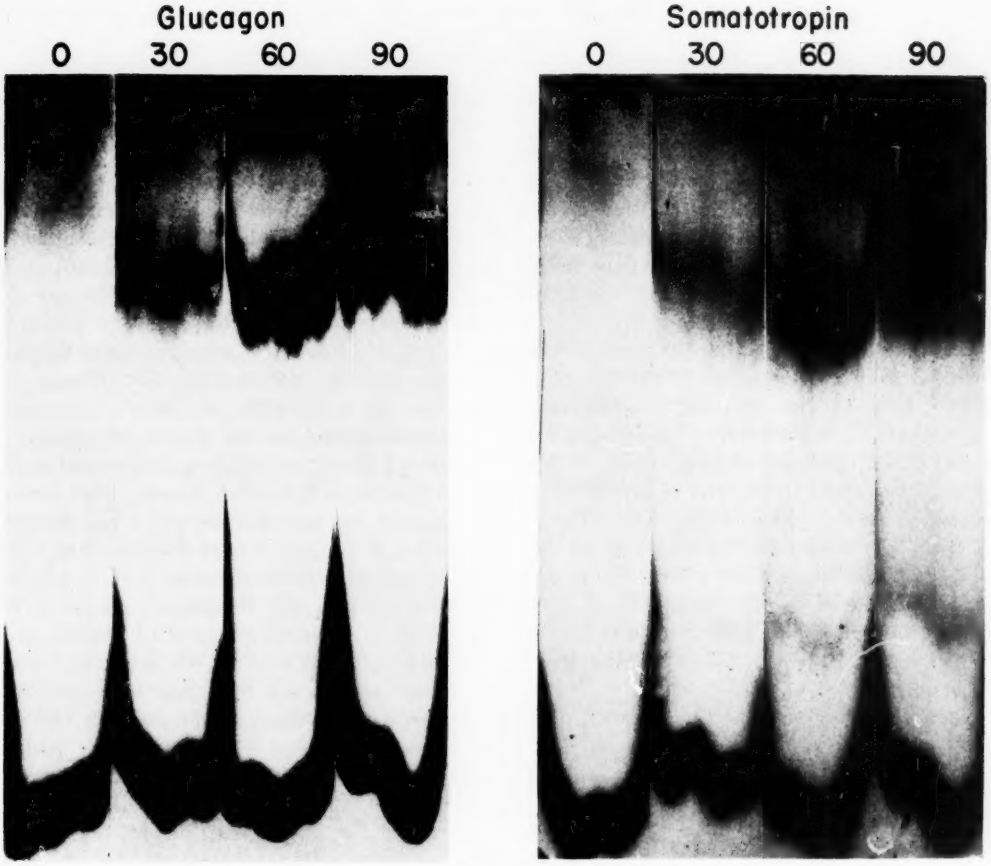

FIG. 5. DEGRADATION OF GLUCAGON AND OF SOMATOTROPIN BY STREPTOKINASE-ACTIVATED PLASMA

Radioautographs of descending chromatograms of aliquots (100 λ) of incubation mixtures removed at designated intervals, streaked on paper and chromatographed as indicated in text. The radioautographs were cut just below the point at which the aliquots were streaked.

with insulin (final concentration, 0.1 μM per ml. incubation mixture) produced an insignificant effect on the degradation of insulin during the two hour incubation period. The maximum change that was found with nine different samples of plasma was 0.9 per cent which is well within the error of the procedure; the mean plus or minus standard error was 0.3 ± 0.4 per cent. Of questionable significance also was the effect of the same concentration of native plasma on the degradation of ACTH and somatotropin (Figure 1). A more significant rate of degradation, however, occurred when glucagon was incubated with samples of the same native plasma.

Incubation with streptokinase alone produced no degradation of the four hormones. In the presence of both plasma and streptokinase there was a marked increase in the rate at which ACTH, somatotropin and especially glucagon were degraded (Figure 1). The degradation of insulin, however, was not influenced appreciably, the maximum increase at the end of two hours being only 1.4 per cent with a mean plus or minus standard error of 0.6 ± 0.5 per cent.

The effect of the incubation of the various proteins (final concentration, 0.1 μM per ml. incubation mixture) with plasminogen alone (final concentration equivalent to 1 ml. plasma per ml. incubation mixture) and with plasminogen plus streptokinase is illustrated in Figure 2.

Plasminogen alone produced no appreciable effect on the destruction of insulin which in six experiments showed a maximum degradation of 1.3 per cent (mean ± S.E., 0.9 ± 0.8 per cent) at the end of the 90 minute incubation period. The presence of streptokinase did not influence the effect of the action of plasminogen on insulin. Even with a 10-fold increase in the concentration of plasminogen or with a prolongation of the incubation period to 72 hours there was no appreciable increase in the quantity of insulin destroyed over that obtained in two hours.

Whereas the plasminogen preparation alone produced a slight destruction of ACTH (mean ± S.E., 1.7 ± 1.6 per cent) and of somatotropin (mean ± S.E., 1.7 ± 1.6), a more significant degradation of glucagon occurred (mean ± S.E., 6.7 ± 2.7 per cent) by the end of the 90 minute incubation period. In contrast to the effect on insulin, the addition of streptokinase to the incubation mixture resulted in a marked acceleration in the rate of degradation of ACTH, somatotropin and glucagon. This effect was inhibited in the presence of crystallized soybean trypsin inhibitor (Figure 2).

The effect of varying the concentration on the degradation of the different substrates by streptokinase-activated euglobulin fraction (equivalent to 1 ml. plasma per ml. incubation mixture) is illustrated in Figure 3. It is apparent that at all concentrations tested, the rate of degradation of each hormone remained essentially constant.

In order to establish that the increase in the TCA soluble radioactive fragments is due to the hydrolysis of the labeled proteins, the effect of the incubation of streptokinase-activated plasminogen (equivalent to 1 ml. plasma per ml. incubation mixture) with the various proteins (1.0 μM per ml. incubation mixture) on the optical density at 280 mμ of the TCA filtrate was studied. It is evident from Figure 4 that concomitant with the amount of protein degraded, as calculated from the per cent of the total radioactivity that became TCA soluble, there was an increase in the optical density. Additional confirmation of proteolysis was obtained from radioautographs of chromatograms of the TCA soluble fraction (Figure 5) prepared according to the technique of Tong, Taurog and Chaikoff (14).

Purified human plasmin, like the streptokinase-activated plasma and the streptokinase-activated plasminogen, produced no appreciable destruc-

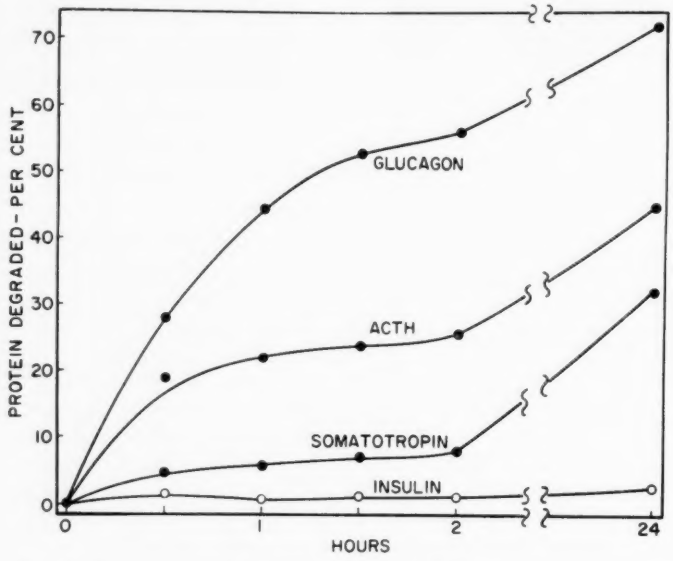

FIG. 6. DEGRADATION OF INSULIN, GLUCAGON, ADRENOCORTICOTROPIN AND SOMATOTROPIN BY PURIFIED HUMAN PLASMIN

The per cent degraded at each interval was computed as indicated in text.

tion of insulin even after incubation for 24 hours. Glucagon and ACTH, however, were rapidly destroyed by the plasmin while somatotropin was destroyed at a slower rate. Typical of the data obtained with the two preparations of purified plasmin used in this study are the data illustrated in Figure 6.

DISCUSSION

The data reported herein reveal that insulin is not degraded *in vitro* by native human plasma, streptokinase-activated plasma, streptokinase-activated plasminogen or purified human plasmin. Glucagon, ACTH and somatotropin, however, are hydrolyzed to some degree by native plasma and at a much greater rate by streptokinase-activated plasma, streptokinase-activated plasminogen and plasmin. The relatively low rate of destruction produced by native plasma does not contradict the conclusions of Reiss, Badrick, Halkerston and Plaice (15) and of Pincus, Hopkins and Hechter (16) that ACTH is inactivated by human plasma. Thus, Pincus and co-workers found that about 90 per cent of the activity of ACTH is destroyed after one hour incubation of 0.002 to 0.004 International unit per ml. of human plasma. This rate of inactivation is within the range of the degradation observed herein since the incubation of approximately five units ACTH with 0.5 ml. plasma for two hours resulted in the degradation of the equivalent of from 0.1 to 0.2 unit.

The conversion of plasminogen to plasmin by streptokinase appears to be relatively specific to human blood (1) in that the plasminogen in native human plasma is readily activated by amounts of streptokinase which are without effect on the plasma of many subhuman species (17). This relative specificity is believed to be due to a "proactivator" which is presumed to be present in large concentrations only in human plasma (1, 18, 19). Accordingly, the destruction of glucagon, ACTH and somatotropin by streptokinase-activated human plasma and streptokinase-activated plasminogen may be due to the resultant plasmin as proposed by White and Gross as the mechanism responsible for the destruction of ACTH by plasma (7). In accord is the inhibitory action of crystallized soybean trypsin inhibitor on streptokinase-activated plasminogen. The relatively low activity of native plasma may be attributed to the spontaneous conversion of some plasminogen to plasmin during the process of drawing the blood and separating the plasma.

The fact that the hydrolytic activity of the streptokinase-activated plasma was not completely inhibited by the quantities of soybean trypsin inhibitor used herein suggests that either the glucagon was not stable under the conditions of the assay or that some other proteolytic enzyme is also present in the plasma. It is pertinent to note, however, that even though a complete inhibition of proteolysis can be produced with larger quantities of soybean inhibitor, the possibility that another proteolytic system is also present is not eliminated because of the fact that soybean trypsin inhibitor is not very specific in its inhibition of proteolytic enzymes.

Plasmin has been shown to hydrolyze synthetic substrates such as arginine and lysine esters (20). The same apparent specificity is suggested by its action on corticotropin A (7) which is split after arginine in the sequence

$$\underset{8}{\text{Arg.}}\ \underset{9}{\text{Try.}}$$

and after lysine in the sequence

$$\underset{15}{\text{Lys.}}\ \underset{16}{\text{Lys.}}\ \underset{17}{\text{Arg.}}\ \underset{18}{\text{Arg.}}\ (\underset{19}{\text{Pro.}}\ \underset{20}{\text{Val.}})$$

Plasmin, however, did not hydrolyze the bond after lysine in position 21

$$(\underset{21}{\text{Lys.}}\ \underset{22}{\text{Val.}})$$

which may reflect a specificity requirement involving the amino-bearing residue of the bond attacked and also explain the failure to hydrolyze the linkage after arginine in position 18. If plasmin has the same type of action on other peptide hormones, then glucagon, the structure of which has been described by Bromer, Sinn, Staub and Behrens (21), may be split after lysine in the sequence

$$\underset{12}{\text{Lys.}}\ \underset{13}{\text{Try.}}$$

but not after arginine in the sequence

$$\underset{17}{\text{Arg.}}\ \underset{18}{\text{Arg.}}$$

Likewise, it could be anticipated that the B chain of insulin (22), if at all hydrolyzed, would split after arginine in the sequence

$$\underset{22}{\text{Arg.}}\ \underset{23}{\text{Gly.}}$$

or after lysine in the sequence

$$\text{--- Lys.}_{29}\ \text{Ala.}_{30}\ \text{---}$$

and release at best only one I^{131} tagged tyrosine residue. The demonstration that insulin is not hydrolyzed by plasmin, however, suggests that the configuration of the intact molecule protects the aforementioned bonds.

As indicated at the outset, exposure to a variety of noxious stimuli may result in the conversion of plasminogen to plasmin *in vivo*. Accordingly, it may be postulated that such exposure may result in an increase in the rate of destruction of circulating glucagon, ACTH and somatotropin. Whether such an increase plays a significant role in the destruction of the various hormones by the intact organism remains unknown.

SUMMARY

Studies with native human plasma, the crude euglobulin fraction (plasminogen) of such plasma, streptokinase-activated plasma, streptokinase-activated plasminogen and purified human plasmin indicate that glucagon, adrenocorticotropin and somatotropin are very susceptible to hydrolysis by plasmin while insulin is resistant to such action.

ACKNOWLEDGMENTS

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REFERENCES

1. Astrup, T. Fibrinolysis in the organism. Blood 1956, **11**, 781.
2. Biggs, R., Macfarlane, R. G., and Pilling, J. Observations on fibrinolysis; experimental activity produced by exercise or adrenaline. Lancet 1947, **1**, 402.
3. Latner, A. L. Anxiety as a cause of fibrinolysis. Lancet 1947, **1**, 194.
4. Tagnon, H. J., Levenson, S. M., Davidson, C. S., and Taylor, F. H. L. The occurrence of fibrinolysis in shock, with observations on the prothrombin time and the plasma fibrinogen during hemorrhagic shock. Amer. J. med. Sci. 1946, **211**, 88.
5. Macfarlane, R. G., and Biggs, R. Fibrinolysis; its mechanism and significance. Blood 1948, **3**, 1167.
6. Kwaan, H. C., and McFadzean, A. J. S. On plasma fibrinolytic activity induced by ischaemia. Clin. Sci. 1956, **15**, 245.
7. White, W. F., and Gross, A. M. Studies on adrenocorticotropin. XIV. Action of bovine fibrinolysin and of liver cathepsin on corticotropin-A: Effect on biological activity. J. Amer. chem. Soc. 1957, **79**, 1141.
8. Milstone, H. A factor in normal human blood which participates in streptococcal fibrinolysis. J. Immunol. 1941, **42**, 109.
9. Kline, D. L. The purification and crystallization of plasminogen (profibrinolysin). J. biol. Chem. 1953, **204**, 949.
10. Sherry, S., and Alkjaersig, N. Biochemical, experimental and clinical studies of proteolytic enzymes: With particular reference to the fibrinolytic enzyme of human plasma. Ann. N. Y. Acad. Sci. 1957, **68**, 52.
11. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A. Distribution and metabolism of I^{131} labeled human serum albumin in congestive heart failure with and without proteinuria. J. clin. Invest. 1955, **34**, 1359.
12. McIlvaine, T. C. A buffer solution for colorimetric comparison. J. biol. Chem. 1921, **49**, 183.
13. Mirsky, I. A., Perisutti, G., and Dixon, F. J. The destruction of I^{131}-labeled insulin by rat liver extracts. J. biol. Chem. 1955, **214**, 397.
14. Tong, W., Taurog, A., and Chaikoff, I. L. Nonthyroglobulin iodine of the thyroid gland. I. Free thyroxine and diiodotyrosine. J. biol. Chem. 1951, **191**, 665.
15. Reiss, M., Badrick, F. E., Halkerston, I. D. K., and Plaice, C. Inactivation of adrenotropic hormone (ACTH) by plasma. Nature 1951, **168**, 206.
16. Pincus, G., Hopkins, T. F., and Hechter, O. An ACTH-inactivating factor in mammalian blood. Arch. Biochem. 1952, **37**, 408.
17. Christensen, L. R. The streptokinase-plasminogen system *in* Streptococcal Infections, M. McCarty, Ed. New York, Columbia University Press, 1954, p. 39.
18. Sherry, S. The fibrinolytic activity of streptokinase activated human plasmin. J. clin. Invest. 1954, **33**, 1054.
19. Müllertz, S. Activation of plasminogen. Ann. N. Y. Acad. Sci. 1957, **68**, 38.
20. Troll, W., Sherry, S., and Wachman, J. The action of plasmin on synthetic substrates. J. biol. Chem. 1954, **208**, 85.
21. Bromer, W. W., Sinn, L. G., Staub, A., and Behrens, O. K. The amino acid sequence of glucagon. Diabetes 1957, **6**, 234.
22. Sanger, F. Fractionation of oxidase insulin. Biochem. J. 1949, **44**, 126.

THE MECHANISM OF POTASSIUM EXCRETION IN THE CHICKEN

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The mechanism of potassium excretion by the kidney has been investigated in a number of species (1, 2). Although much information has been deduced from these studies, including an analysis of the probable intra- and peritubular events involved, the interpretation of results has often been complicated by extrarenal effects induced by the experimental procedures. In order to obviate these difficulties and to provide information on the role of alterations in the peritubular concentrations of electrolytes in the process, some of the phenomena relating to the renal excretion of potassium have been reinvestigated in a direct fashion in the chicken.

Chickens possess a functioning renal-portal circulation (3). Blood from the leg vein drains directly into the postglomerular peritubular vessels on that side, and then enters the systemic circulation via the vena cava. The glomerulus is thereby bypassed in the first circulation. It is possible to investigate agents which exert effects on tubule cells by administering the test substance in one leg and comparing urine from the kidney of the infused side with that from the contralateral kidney which serves as a control. This procedure, originally devised by Sperber (4), minimizes the role of glomerular filtration. It permits one to achieve concentrations of test substance in peritubular blood of the experimental side which would be lethal in the systemic circulation. Furthermore the concentration of test substance on the injected side is always greater than in peritubular blood of the contralateral control kidney.

METHODS

Rhode Island Red hens weighing approximately 2 to 4 Kg. each were used in all studies. The procedure was essentially that of Sperber (4). Animals were suspended in the upright position in a cloth sling. Food was withdrawn for 12 to 18 hours prior to all studies. To insure adequacy of urine flow in control periods, 80 to 100 ml. of water was administered by crop tube at the beginning of each study.

All solutions were administered intravenously by means of a Bowman constant infusion pump. Infusions into the renal-portal venous system of the experimental side were introduced by means of a polyethylene catheter in the saphenous vein of that side. Infusions directly into the systemic circulation were administered through the wing vein.

Urine was collected separately from each ureter by means of small polyethylene funnels sewn directly over the individual ureteral openings in the cloaca. Urine from the kidney of the injected side is referred to in tables and figures as being from the "experimental side"; that from the contralateral kidney, the "control side." Blood for analyses was drawn into oiled heparinized syringes at the midpoint of alternate periods from either the leg, wing or neck veins.

Glomerular filtration rate was estimated by the clearance of inulin. The clearance of inulin was independent of the site of infusion when the inulin was infused into either of the leg veins or directly into the systemic circulation via the wing vein.

Renal plasma flow was measured by the clearance of para-aminohippurate (PAH). This averaged approximately 50 ml. per minute per side in 2 Kg. chickens. Solutions of PAH were injected into the wing vein.

Inulin was measured in plasma and urine by the method of Walser, Davidson and Orloff (5), PAH in either whole blood or plasma and urine by the method of Bratton and Marshall (6). Sodium and potassium in plasma and urine were determined by flame photometry, urine chloride by a modified Volhard titration or potentiometry (7). The pH of urine was measured in a Beckman Model G pH meter at room temperature or in a Cambridge Research model pH meter at 37° C. Total CO_2 was determined by the method of Van Slyke and Neill (8).

The peritubular concentration of potassium was estimated as the sum of the peripheral venous potassium concentration plus

$$\frac{\text{potassium injected } (\mu\text{M per minute})}{\text{renal plasma flow (ml. per minute)}}$$ [1]

[1] The calculation of the peritubular concentration of potassium is only a first approximation. It has not been possible to determine with any degree of accuracy the precise volume flow of peritubular plasma; consequently,

TABLE I

Effect of K_2SO_4 on electrolyte excretion

Time *min.*	Plasma K^+ *mEq./L.*	Control kidney GFR *ml./min.*	pH	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	$U_{Cl}V$ *μEq./min.*	E/F*	P-T† K^+ *mEq./L.*	Experimental kidney GFR *ml./min.*	pH	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	$U_{Cl}V$ *μEq./min.*	E/F*
−30	Infuse 1.5 mg./min. inulin in 5% dextrose in H_2O at .22 ml./min. into wing vein. Priming inulin 160 mg.													
0–9	3.3	7.0	5.76	2	5	.3	.21	3.3	6.5	5.83	2	5	.5	.22
9–17	3.3	6.0	5.74	2	4	.1	.19	3.3	6.0	5.85	2	4	.3	.18
33–49	3.3	6.0	5.48	4	4	.1	.19	3.3	5.1	5.58	4	4	.1	.21
50	Infuse 100 μM/min. of K_2SO_4 into leg vein (experimental side)													
55–65	4.3	4.8	5.05	12	9	1	.42	7.7	4.1	6.06	15	29	3	1.67
65–78	4.9	4.3	5.46	21	30	2	1.44	8.5	4.0	6.76	28	64	27	3.26
78–89	5.2	5.2	5.71	30	50	10	1.83	8.8	4.8	6.88	52	87	45	3.48
89–99	5.5	5.1	5.19	36	55	14	1.98	9.5	5.4	6.19	39	81	36	2.72
99–109	5.8	5.2	5.74	38	70	19	2.31	10.0	5.0	6.69	43	91	34	3.12
110–120	6.1	6.2	5.75	49	84	28	2.30	11.1	5.2	6.82	52	96	39	3.03

* E/F is the ratio of excreted to filtered potassium.
† P-T refers to peritubular plasma K^+ concentration.

RESULTS

The effect of the infusion of potassium

The infusion of potassium into one leg vein uniformly resulted in an increase in the rates of excretion of potassium, sodium and chloride. The effects were more marked on the side of the infusion (experimental kidney, Table I), due to the higher concentration of potassium in the peritubular blood of that side. In the study illustrated, urine pH rose and remained elevated on the experimental side. In others (see below) an initial unilateral rise in urine pH was followed by a decrease to or below control values. The excretion of potassium exceeded that filtered in Periods 4 through 9 on the experimental side and Periods 5 through 9 on the control side, evidence that potassium is secreted in the chicken as in other species examined. However unlike the situation in dog and man in which prior administration of potassium for a few days is generally necessary to elicit net secretion, the infusion of potassium into the leg vein of a chicken uniformly evokes this response.

Figure 1 illustrates the relationship between potassium excretion and the concentration of potassium in peritubular plasma. It can be seen that the rate of excretion is not a linear function of plasma concentration. This is also evident in Table I. At high plasma concentrations potassium excretion approaches a maximal rate which is relatively constant and only minimally affected by acute alterations in tubular load. In the study illustrated (Figure 1), 100 μM per minute of potassium sulfate was injected into the leg vein. Potassium excretion increased rapidly at first, reached a maximum of approximately 65 μEq. per minute and remained at this value despite a progressive increase in the calculated concentration of potassium in peritubular plasma from 9 to 12 μEq. per ml. No significant change in renal blood flow occurred on the experimental side. Blood flow averaged 53.3 ml. per minute in the control periods and 52.1 during the administration of potassium. The rate of potassium excretion rose on the control side as well, reaching approximately the same rate when the peripheral venous plasma concentration was also 9 μEq. per ml, denoting that the tubular load on the control side was now equivalent to that at which "saturation" was achieved on the experimental side. Similar results were noted in three other studies. The rate of change of potassium excretion decreased markedly at high levels, approaching a limiting value in all studies. Changes in renal blood flow on this side were variable. In one, blood flow rose 25 per cent during the course of the infusion, in two a significant decrease occurred (43 and 9 per cent, respectively). Potassium excretion continued to rise on the control side despite similar changes in blood flow. Acute increases in the rate of potassium adminis-

total renal plasma flow has been used as an estimate of the minute volume of distribution of injected potassium.

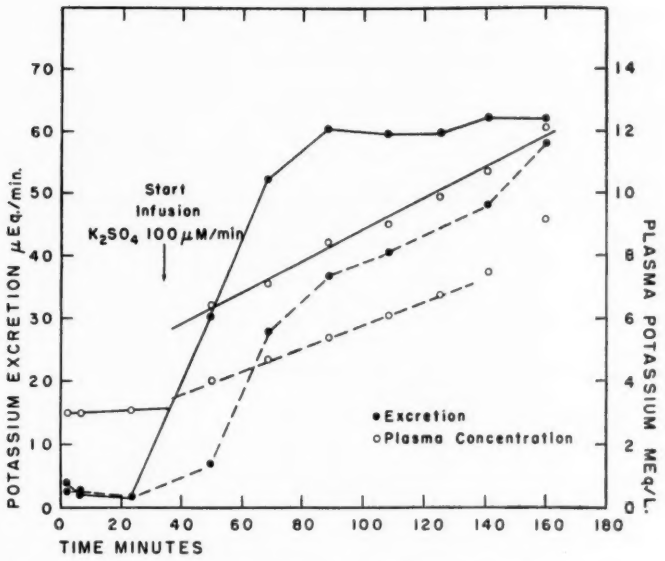

FIG. 1. THE RELATIONSHIP BETWEEN THE EXCRETION OF POTASSIUM AND THE PERITUBULAR CONCENTRATION OF POTASSIUM IN PLASMA

Continuous lines are for the experimental kidney; broken lines, control kidney.

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tration from 200 to 300 μEq. per minute did not alter the steady state excretion by more than 10 μEq. per minute. It is apparent from these data that the accumulation of potassium in urine is not a consequence of simple diffusion from plasma. At least one step in the process is apparently rate-limiting and capable of saturation

In the experiments noted in Table I and Figure 1, potassium was administered as the sulfate salt. Results were qualitatively the same with

TABLE II

Effect of Na_2SO_4 on electrolyte excretion

Time *min.*	Control kidney			Experimental kidney		
	pH	$U_{Na}V$ *μEq./min.*	U_KV	pH	$U_{Na}V$ *μEq./min.*	U_KV
−100	Infuse 1.5 mg./min. inulin in 5% dextrose in H_2O at 0.25 ml /min. into wing vein. Priming inulin 150 mg.					
100–116	5.47	6	5	5.36	7	5
116–131	5.38	10	6	5.08	6	4
131–156	5.16	10	4	5.22	10	4
157	Infuse 100 μM/min. Na_2SO_4 into leg vein (experimental side)					
157–184	4.71	12	4	4.68	14	5
185–203	4.85	14	4	4.70	16	5
203–231	4.73	18	5	4.70	11	3
231–244	4.74	21	5	4.67	20	5
249–272	4.79	16	3	4.72	27	6
272–284	4.92	23	5	4.59	21	4

TABLE III

Effect of acetazoleamide on electrolyte excretion

Time min.	Plasma K mEq./L.	Control kidney GFR ml./min.	Control kidney pH	Control kidney $U_{Na}V$ μEq./min.	Control kidney U_KV μEq./min.	Experimental kidney Peritubule K mEq./L.	Experimental kidney GFR ml./min.	Experimental kidney pH	Experimental kidney $U_{Na}V$ μEq./min.	Experimental kidney U_KV μEq./min.
0	Infuse 150 mg. inulin prime									
0	Infuse 1.5 mg./min. inulin, 100 μM/min. K_2SO_4 into leg vein (experimental side)									
9–19	5.25	4.2	5.94	17	40	7.9	4.0	7.32	34	73
20–40	5.9	3.6	6.63	8	41	9.4	3.0	7.44	26	67
43–56	6.6	3.2	5.98	11	47	11.4	3.0	7.16	23	80
57–63	7.0	3.3	6.23	15	59	12.0	3.1	6.88	31	81
63–69	7.2	3.3	6.04	19	57	12.2	3.3	7.09	41	86
69–77	7.45	3.4	5.83	17	65	12.3	3.3	6.88	34	87
78–86	7.7	3.3	6.13	20	68	12.6	3.2	7.11	35	89
84	Infuse acetazoleamide, 5 mg./Kg./hr. into leg vein (experimental side)									
86–92	7.95	3.4	6.53	37	75	11.7	4.0	7.15	50	92
94–101	8.25	3.5	6.78	43	94	13.3	4.2	7.07	46	93
101–112	8.55	2.8	6.73	40	82	13.8	3.2	7.11	43	87
113–121	8.8	3.4	6.54	33	83	13.8	3.4	7.01	41	93
122–129	9.05	3.8	6.73	48	95	14.2	3.5	7.12	51	99

potassium chloride although the absolute rates of potassium excretion were not as great. The administration of equimolar amounts of sodium sulfate into one leg (Table II) resulted in no unilateral changes in urine composition; nor was any effect on potassium excretion demonstrable, indicating 1) the absence of a specific effect of sulfate ion on potassium transport, and 2), indirectly, that sulfate ion enters the urine largely, if not entirely, through the glomerulus. Similar results were noted in two other studies

The administration of a carbonic anhydrase inhibitor, acetazoleamide, elicits a response in the chicken (9) essentially similar to that observed in dog and man (10, 11). Five mg. per Kg. per hour of acetazoleamide increases the excretion of potassium, sodium and bicarbonate, and elevates urine pH during the infusion of sodium chloride. Table III illustrates the effect of this dose on potassium excretion when the latter has been augmented to high rates by the prior administration of potassium sulfate. The administration of acetazoleamide was associated with an increase in potassium excretion on the experimental side of only 10 μEq. per minute, a rise no greater than that occasionally observed in studies in which the rate of potassium infusion is acutely increased from 200 to 300 μEq. per minute (see above). A somewhat greater augmentation of potassium excretion was observed on the control side. In one other study acetazoleamide did not elevate potassium excretion on the experimental side above the "maximal" rate induced by potassium sulfate administration despite a clearcut rise on the control side. It may be concluded that acetazoleamide is incapable of appreciably affecting the transport system for potassium once it has been "saturated" by prior injection of potassium.[2]

Alkalinization of the urine following the administration of potassium is observed in the chicken (Table I, Figure 2 [3]) as in dog and man (10, 12, 13). The decrease in hydrogen ion concentration is associated with a rise in potassium excretion and is presumably a consequence of the competitive nature of the mechanism by which hydrogen and potassium ions are secreted in exchange for sodium. This has been discussed in detail elsewhere (10). The importance of one other factor, the filtered load of bicarbonate, in the determination of urine pH is illustrated in Figure 2. Since bicarbonate reabsorption is effected by hydrogen ion secretion

[2] It was not possible to demonstrate a unilateral effect of the inhibitor on urine composition when the drug was administered continuously. However, if urine was collected continuously by catheter irrigation, transient (10 to 15 minute) unilateral alkalinization on the infused side was noted when a single injection of 0.5 mg. of acetazoleamide was administered. It is apparent from this that the drug need not be filtered to exert its effect and may penetrate tubule cells from the contraluminal side.

[3] The data are from the same study as Figure 1.

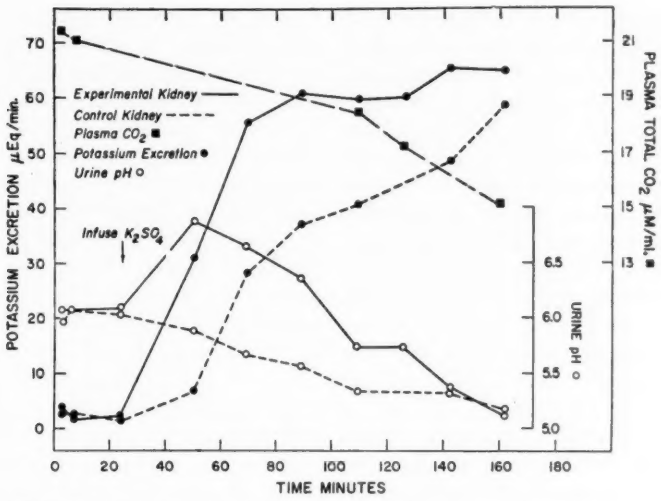

FIG. 2. EFFECT OF THE INFUSION OF K_2SO_4 INTO THE LEG VEIN OF A CHICKEN ON THE EXCRETION OF POTASSIUM, URINE pH, AND PLASMA TOTAL CO_2 CONCENTRATION

(14), a reduction in plasma bicarbonate insofar as it decreases the load of bicarbonate claiming reabsorption, will diminish the total hydrogen ion requirement for this process. Consequently a secondary fall in urine pH, as noted in Figure 2, may supervene as a result of the development of metabolic acidosis, despite a decrease in hydrogen ion secretion. Similar changes in plasma bicarbonate concentration and urine pH following injection of potassium have been observed in the rat and dog (15, 16). Metabolic acidosis presumably develops as a result of the extrusion of hydrogen ions from muscle and other nonrenal tissue cells in exchange for entering potassium (17–19) as well as to the loss of bicarbonate in the urine (20).

Effect of alterations in peritubular pCO_2

Respiratory modifications of acid-base balance are known to affect the excretion of potassium and hydrogen ions (21–24). An increase in urine hydrogen ion concentration and a de-

TABLE IV

Effect of local rise in pCO_2 on K^+ excretion and urine pH

Time *min.*	Plasma K^+ *mEq./L.*	Control kidney: Plasma flow *ml./min.*	Control kidney: pH	Control kidney: U_KV *μEq./min.*	Experimental kidney: P-T* K^+ *mEq./L.*	Experimental kidney: Plasma flow *ml./min.*	Experimental kidney: pH	Experimental kidney: U_KV *μEq./min.*
−50	Infuse 200 μEq./min. K_2SO_4 into leg vein (experimental side)							
0–8	4.3	44	4.51	49	9.6	37	5.93	72
8–16	5.3	47	4.46	56	10.6	37	6.23	74
16–25	6.5	47	4.59	49	12.9	31	5.67	69
26–30	7.6	38	4.67	49	14.8	28	6.27	68
30	Infuse 300 μM/min. acetic acid into leg vein. Continue K_2SO_4							
34–44	8.3	36	5.05	52	13.6	38	5.18	45
44–53	8.8	31	5.21	54	14.5	35	4.95	39
54–63	9.3	32	4.85	58	15.2	34	4.92	38
63–70	9.6	35	4.72	47	15.2	36	4.66	35

* P-T refers to peritubular plasma K^+ concentration.

crease in potassium excretion have been observed in respiratory acidosis, and the converse in alkalosis. The effects are thought to be secondary to alterations in renal tubule cell pCO_2 conditioned by parallel changes in the pCO_2 of renal blood and interstitial tissue. The rise in intracellular pCO_2 and hydrogen ion concentration in respiratory acidosis accelerates the transfer of hydrogen ion into urine, inhibiting the transfer of potassium in the process. Analogous effects due to local changes in peritubular pCO_2 are illustrated in the experiment detailed in Table IV. Potassium sulfate was injected into one leg vein. This, as always, resulted in a marked augmentation of potassium excretion on that side and a fall in urine hydrogen ion concentration. After 80 minutes, 300 μM per minute of acetic acid was added to the potassium sulfate infusion. A prompt decrease in potassium excretion and a rise in urine hydrogen ion concentration occurred on the experimental side. Although acidosis (plasma total CO_2 17 to 18 mEq. per L.) due to the administration of potassium was present prior to the infusion of acetic acid, the urinary effects are clearly due to a rise in the pCO_2 of the peritubular tissue and are not conditioned by changes in systemic acid-base balance. Subsequent conversion of acetate to bicarbonate apparently prevents the development of a greater degree of metabolic acidosis. Similar results were observed in five other studies. The rise in H_2CO_3 concentration and pCO_2 of peritubular plasma is due to the interaction of acetic with bicarbonate in blood. The CO_2 excess escapes into the first capillary bed to which the blood is delivered, that of the peritubular region, and since CO_2 is freely diffusible there is a concomitant rise in tubule cell pCO_2 and hydrogen ion concentration. Addition of hydrochloric acid to renal portal venous blood evokes the same response. Local tubule cell "respiratory" acidosis occurs as with acetic acid, although in this instance it is probable that a greater degree of systemic metabolic acidosis develops.

The converse experiment, addition of sodium hydroxide to saphenous vein blood to lower the pCO_2 of the cells, was generally unsatisfactory. The experiments were frequently lethal and it was not possible to demonstrate an unequivocal unilateral effect. Furthermore, a marked rise in plasma bicarbonate (and therefore filtered load of bicarbonate) resulted in bilateral alkalinization of the urine and kaluresis, not unlike that noted when an infusion of sodium bicarbonate is administered into wing vein.

Unilateral alkalinization of the urine is not observed if sodium bicarbonate is introduced into the renal portal circulation although this has

TABLE V

Effect of Salyrgan® on electrolyte excretion

Time *min.*	Control kidney				Experimental kidney			
	Filt.* K^+	U_KV	$U_{Na}V$	$U_{Cl}V$	Filt. K^+	U_KV	$U_{Na}V$	$U_{Cl}V$
		μEq./min.				*μEq./min.*		
−20	Infuse 100 μM/min. KCl into leg vein (experimental side)							
0–30	18	9	3	10	17	22	3	20
31–46	18	9	5	12	18	38	4	33
47–70	16	10	6	14	18	37	4	29
88	Infuse 0.3 ml. Salyrgan® followed by .005 ml./min. into wing vein. Continue KCl							
82–101	19	8	15	14	20	18	20	22
101–108	18	6	25	26	22	12	31	37
108–113	20	5	37	37	22	11	42	51
113–117	23	5	49	49	23	11	57	60
127	Inject 20 mg. dimercaprol (BAL) intramuscularly							
125–130	22	22	25	36	25	34	32	—
131–138	22	20	15	27	22	32	23	49
138–145	19	18	9	19	22	31	15	39

* Filtered K^+ = GFR × plasma K^+ concentration.

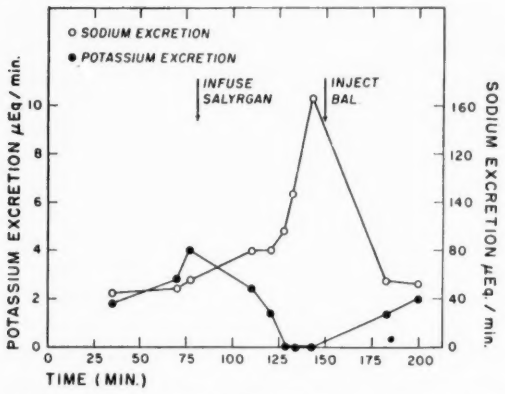

FIG. 3. EFFECT OF SALYRGAN® ON THE TOTAL URINARY EXCRETION OF SODIUM AND POTASSIUM IN THE CHICKEN

been reported in the frog (25). Instead, bilateral alkalinization and kaluresis develop when the filtered load of bicarbonate rises. This occurs despite the fact that the pCO_2 on the infused side is undoubtedly somewhat greater than on the control side. However, if the pCO_2 is further elevated by addition of acetic acid, the usual reduction in potassium excretion is noted.

Effect of Salyrgan®

Direct and reversible inhibition of potassium excretion by mercurial diuretics has been observed repeatedly (2, 26). Whether this is due to enhanced reabsorption of potassium or diminished secretion or both has not been entirely clear. Furthermore, mercury does not depress potassium excretion under all circumstances in these species, complicating the interpretation of results. These difficulties are not encountered in the chicken. The filtration fraction is so low (0.05) that the potential contribution of filtered and nonreabsorbed potassium to that excreted is minimal. Also, mercury uniformly depresses potassium excretion in this animal, irrespective of the initial rate of excretion.

The effect of Salyrgan® is illustrated in Table V. Potassium excretion was initially augmented by the injection of potassium chloride. The injection of 0.3 ml. Salyrgan® caused a rapid fall in potassium excretion which was reversed by 20 mg. dimercaprol [BAL (British anti-Lewisite)]. The decrement in potassium excretion was greater than could be accounted for by increased reabsorption, clearly denoting that the mercurial interfered with the secretory process.

In contrast to studies in dog and man in which mercury increases potassium excretion when it is initially low (26), in the chicken Salyrgan® interferes with excretion under these circumstances as well. In the study illustrated in Figure 3, potassium virtually disappeared from the urine following Salyrgan®. This observation limits the effect of mercury in this species to interference with secretion. Furthermore it is consistent with the view that all filtered potassium is reabsorbed, as had been suggested on the basis of other data in the dog (27). The low basal rate of potassium excretion in the control periods therefore probably represents secreted potassium only.

DISCUSSION

It has been suggested in previous publications that potassium and hydrogen ions are secreted into urine in exchange for sodium and that these ions compete for some portion of the exchange process by which sodium is reabsorbed (10). The data obtained in these studies constitute additional support for this thesis. Furthermore they afford direct and unequivocal evidence that the transfer of potassium and hydrogen ions into urine is conditioned by alterations in the peritubular concentrations (and therefore presumably the renal tubule cell concentrations) of these ions and not necessarily by extrarenal (systemic) changes in acid-base balance. The latter are of importance, however, insofar as they affect the filtered load and tubule cell concentrations of electrolytes.

Linked transfer of hydrogen, potassium and sodium ions is thought to be effected by specific and reversible combination with a carrier. Although no direct evidence for this exists, the results of these and other studies are consistent with this view. The observations that the process may be saturated (presumably indicating enzyme or carrier limitation), that it is subject to competitive inhibition, and finally that it may be interfered with in a reversible manner by a pharmacologic agent, are best interpreted within the framework of the carrier hypothesis. These arguments will be discussed separately, but first

it should be noted that none of the data affords any insight into the precise nature of the mechanism, that is whether the ions are actively or passively transported. Neither the demonstration of net potassium secretion nor the observation that the concentration of potassium in urine exceeds that of plasma is sufficient evidence to conclude that potassium is transported by an active process. Demonstration of active transport, movement of an ion against its electrochemical gradient (28), requires knowledge of the chemical gradient in the pertinent area and the transtubular potential. Despite the lack of this information, it is relatively certain that linked transfer by a carrier does occur and that at least one of the cations involved is transported against its electrochemical gradient.

It is clear from the experiments illustrated in Table I and Figure 1 that the excretion of potassium is a function of its peritubular concentration. Since the filtered load of potassium is equal on both sides, the unilateral changes noted in these studies can only be due to alterations in tubule cell transport induced by the higher concentration of potassium. Of greater significance in the context of this discussion is the observation that at high concentrations of peritubular potassium the relationship between urinary excretion and plasma concentration is no longer linear (Figure 2). Excretion clearly approaches a maximal rate, indicating that the transport mechanism may be saturated and that passive diffusion from plasma to urine is not a rate-limiting step in the process. These data constitute strong support for the view that reversible combination with a carrier is involved in potassium transport and that association and dissociation of the carrier-cation complex is rate-limiting. The pertinence of a tubular transfer maximum in the chicken insofar as it relates to the mechanism of potassium transport in other species is not invalidated by the uniqueness of the finding, since only in this preparation is it possible to achieve the concentration of potassium in peritubular plasma necessary to saturate the process.

The reciprocal changes in hydrogen and potassium excretion induced by administration of potassium salts, acetazoleamide or acetic acid are indicative of the competitive nature of the transport process under study. Although similar conclusions had been derived from data in other species (10), the present studies clearly limit the locus of action of the changes responsible for the urinary effects. It is probable that the relationship between the concentrations of the cations in peritubular plasma (and presumably in tubule cells), rather than a reciprocal change in their concentrations, conditions the urinary response (29). Thus, although increased potassium excretion and interference with acidification following the infusion of potassium are probably associated with a reciprocal change in the intracellular concentrations of potassium and hydrogen ions, similar urinary effects due to acetazoleamide are most likely due to intracelluar alkalinization alone. Conversely, aciduria and decreased potassium excretion following introduction of acetic acid are a consequence of an increase in the pCO_2 and hydrogen ion concentration of tubular cells. It is unlikely that cell potassium is appreciably altered in this situation. The hypothetical carrier is assumed to form a complex to a lesser or greater extent with hydrogen or potassium as a function of the intracellular ratio of these ions, the relative preponderance of either ion within the tubular cell determining which is preferentially secreted in exchange for sodium.

The effect of the unilateral infusion of sodium bicarbonate is somewhat anomalous. Rather than observing unilateral alkalinization, as had been reported in the frog (25), bilateral alkalinization and kaluresis occur in association with a rise in filtered bicarbonate. The result differs in no way from the response to the systemic infusion of bicarbonate in either chicken or dog. Were the cell membrane impermeable to bicarbonate, as may be inferred from the data of Wallace and Hastings (30) in muscle, the absence of a unilateral effect would be understandable. However it would then be difficult to account for bilateral alkalinization and kaluresis in the context of the present hypothesis regarding competition between hydrogen and potassium ions. Furthermore the pCO_2 of peritubular plasma is elevated when bicarbonate is added to renal portal blood, and this should, if anything, elicit the reverse: acidification and a fall in potassium excretion. If on the other hand one assumes, on the basis of the observation of bicarbonate uptake by cortical slices by Anderson and Mudge (31), that bi-

carbonate does in fact penetrate the tubule cells, it is possible that the rise in pCO_2 is sufficient (despite an increase in cell bicarbonate) to augment hydrogen ion secretion unilaterally and to mask any effect on urine pH and potassium excretion produced by the transfer of bicarbonate into cells. On the other hand, as previously noted, when bicarbonate is infused into the wing vein of the chicken, bilateral kaluresis and alkalinization occurs. Since the rise in pCO_2 under these circumstances may be less due to interposition of the lungs, it is possible that intracellular alkalinization may be implicated as the factor responsible for the urinary changes.

Alternatively none of the effects noted in the urine need necessarily derive from changes in intracellular composition. Merely increasing the load of sodium presented to the site in the distal segment where potassium is transferred in exchange for sodium will augment potassium excretion. This occurs in other forms of solute diuresis and if secondary to a rise in filtered bicarbonate, the increased concentrations of sodium and bicarbonate delivered to the distal segment could conceivably account for the alkalinization and kaluresis.

In any event the results of the administration of potassium, and the inhibitory effect of a rise in cell pCO_2 on potassium excretion, may be viewed as evidence that the transport system is subject to competitive inhibition not unlike that which would be predicted were reversible combination of either ion with a carrier involved in the exchange process.

Mercurial diuretics, as noted earlier, had been thought to affect both the reabsorption and secretion of potassium in dog and man (2). This was based on the divergent effects of the drug on potassium excretion dependent on the initial level of excretion. Recently evidence has been presented demonstrating that the drug interferes with secretion alone (27). The data obtained in these studies may be interpreted as indicating that the only effect of mercury in the chicken is to interfere with the secretory process. Furthermore the complete abolition of potassium excretion observed (Figure 3) supports the view that all filtered potassium is reabsorbed. The rise in potassium excretion noted after mercury in the dog, when the initial rate of potassium excretion is low, may be a reflection of the increased load of sodium presented to a partially inhibited but unsaturated exchange site. The increased load of sodium provides more opportunity for exchange, even though the process proceeds at less than maximal efficiency.

CONCLUSIONS

The secretion of potassium into urine is inversely related to that of hydrogen ion in the chicken as in other species. Alterations in the secretory rates of potassium and hydrogen ions are mediated by changes in the peritubular (and/or the renal intracellular) concentrations of these ions and are not necessarily related to changes in systemic acid-base balance.

The secretory mechanism for potassium may be saturated, is subject to competitive inhibition, and is interfered with in a reversible fashion by mercury, a pharmacologic inhibitor.

The data have been interpreted as indicating that linked transfer of potassium, hydrogen and sodium ions is probably accomplished by a carrier mechanism. Association and dissociation of the hypothetical cation-carrier complex is apparently rate-limiting in the transport process. Preferential association of either potassium or hydrogen ions with the carrier may be a function of the relative preponderance of one or the other ion within the tubule cell.

REFERENCES

1. Berliner, R. W., Kennedy, T. J., Jr., and Hilton, J. G. Renal mechanisms for excretion of potassium. Amer. J. Physiol. 1950, **162**, 348.
2. Mudge, G. H., Ames, A., III, Foulks, J., and Gilman, A. Effect of drugs on renal secretion of potassium in the dog. Amer. J. Physiol. 1950, **161**, 151.
3. Sperber, I. Investigations on the circulatory system of the avian kidney. Zool. Bidr. Uppsala 1948, **27**, 429.
4. Sperber, I. A new method for the study of renal tubular excretion in birds. Nature (Lond.) **1946**, **158**, 131.
5. Walser, M., Davidson, D. G., and Orloff, J. The renal clearance of alkali-stable inulin. J. clin. Invest. 1955, **34**, 1520.
6. Bratton, A. C., and Marshall, E. K., Jr. A new coupling component for sulfanilamide determination. J. biol. Chem. 1939, **128**, 537.
7. Cotlove, E., Trantham, H. V., and Bowman, R. L. An instrument and method for automatic, rapid, accurate, and sensitive titration of chloride in biologic samples. J. Lab. clin. Med. 1958, **51**, 461.

8. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
9. Wolbach, R. A. Renal regulation of acid-base balance in the chicken. Amer. J. Physiol. 1955, **181**, 149.
10. Berliner, R. W., Kennedy, T. J., Jr., and Orloff, J. Relationship between acidification of the urine and potassium metabolism; effect of carbonic anhydrase inhibition on potassium excretion. Amer. J. Med. 1951, **11**, 274.
11. Friedberg, C. K., Halpern, M., and Taymor, R. The effect of intravenously administered 6063, the carbonic anhydrase inhibitor, 2 acetylamino-1,3,4-thiadiazole-5-sulfonamide, on fluid and electrolytes in normal subjects and patients with congestive heart failure. J. clin. Invest. 1952, **31**, 1074.
12. Winkler, A. W., and Smith, P. K. Renal excretion of potassium salts. Amer. J. Physiol. 1942, **138**, 94.
13. Loeb, R. F., Atchley, D. W., Richards, D. W., Benedict, E. M., and Driscoll, M. E. On the mechanism of nephrotic edema. J. clin. Invest. 1932, **11**, 621.
14. Pitts, R. F., and Lotspeich, W. D. Bicarbonate and the renal regulation of acid base balance. Amer. J. Physiol. 1946, **147**, 138.
15. Leonard, E., and Orloff, J. Regulation of ammonia excretion in the rat. Amer. J. Physiol. 1955, **182**, 131.
16. Roberts, K. E., Magida, M. G., and Pitts, R. F. Relationship between potassium and bicarbonate in blood and urine. Amer. J. Physiol. 1953, **172**, 47.
17. Darrow, D. C., Schwartz, R., Iaunucci, J. F., and Coville, F. The relation of serum bicarbonate concentration to muscle composition. J. clin. Invest. 1948, **27**, 198.
18. Cooke, R. E., Segar, W. E., Cheek, D. B., Coville, F. E., and Darrow, D. C. The extrarenal correction of alkalosis associated with potassium deficiency. J. clin. Invest. 1952, **31**, 798.
19. Orloff, J., Kennedy, T. J., Jr., and Berliner, R. W. The effect of potassium in nephrectomized rats with hypokalemic alkalosis. J. clin. Invest. 1953, **32**, 538.
20. Bourdillon, J. Distribution in body fluids and excretion of ingested ammonium chloride, potassium chloride, and sodium chloride. Amer. J. Physiol. 1937, **120**, 411.
21. Elkinton, J. R., Singer, R. B., Barker, E. S., and Clark, J. K. Effects of acute respiratory acidosis on electrolyte excretion in man. Fed. Proc. 1953, **12**, 38.
22. Dorman, P. J., Sullivan, W. J., and Pitts, R. F. The renal response to acute respiratory acidosis. J. clin. Invest. 1954, **33**, 82.
23. McCance, R. A., and Widdowson, E. M. The response of the kidney to an alkalosis during salt deficiency. Proc. roy. Soc. B 1936, **120**, 228.
24. Stanbury, S. W., and Thomson, A. E. The renal response to respiratory alkalosis. Clin. Sci. 1952, **11**, 357.
25. Chambers, R., and Kempton, R. T. The elimination of neutral red by the frog's kidney. J. cell. comp. Physiol. 1937, **10**, 199.
26. Berliner, R. W., and Kennedy, T. J., Jr. Renal tubular secretion of potassium in the normal dog. Proc. soc. Exp. Biol. (N. Y.) 1948, **67**, 542.
27. Davidson, D. G., Levinsky, N. G., and Berliner, R. W. Maintenance of potassium excretion despite reduction of glomerular filtration during sodium diuresis. J. clin. Invest. 1958, **37**, 548.
28. Ussing, H. H. Transport of ions across cellular membranes. Physiol. Rev. 1949, **29**, 127.
29. Orloff, J. The role of the kidney in the regulation of acid-base balance. Yale J. Biol. Med. 1956, **29**, 211.
30. Wallace, W. M., and Hastings, A. B. The distribution of bicarbonate ion in mammalian muscle. J. biol. Chem. 1942, **144**, 637.
31. Anderson, H. M., and Mudge, G. H. The effect of potassium on intracellular bicarbonate in slices of kidney cortex. J. clin. Invest. 1955, **34**, 1691.

STUDIES ON THE BIOGENESIS AND METABOLISM OF NOREPINEPHRINE IN PATIENTS WITH PHEOCHROMOCYTOMA *

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Discovery of the physiologic importance of norepinephrine and its role as a chemical mediator in the sympathetic nervous system is of relatively recent origin (1). Studies in experimental animals have led to rapid advances in our knowledge of the biochemical processes involved in the formation and metabolism of this amine. It seemed important to confirm and extend these studies in man and to establish their clinical significance. Such investigations are difficult to perform because of the small amounts of norepinephrine normally found in the blood and urine and the fact that fresh human tissue is not easily obtained for enzymatic study. Patients with pheochromocytoma provided a satisfactory solution to this problem since the tumor usually produces large amounts of norepinephrine and is a tissue which can be studied enzymatically after surgical removal. During the past five years, a sufficient number of patients with pheochromocytoma as well as specimens of tumor and urine were made available to enable us to demonstrate a number of the catalysts and intermediates involved in the formation and metabolism of norepinephrine in man and to estimate the rate of its synthesis by the tumors.

MATERIALS AND METHODS

Specimens of acidified urine, and frozen plasma and tumor were received from many sources for assay of catecholamines. However, most of the biochemical studies to be reported were done on seven patients.[1] Tumors from these patients were placed in cracked ice or frozen upon surgical removal for subsequent chemical and enzymatic studies.

Chemical compounds. L-norepinephrine-bitartrate, L-epinephrine-bitartrate and 3,4-dihydroxybenzoic acid were obtained from commercial sources. The 3,4-dihydroxy-DL-phenylalanine-3-C^{14} and DL-epinephrine-2-C^{14}-bitartrate were supplied by Nuclear Chemical and Instrument Company. The 3,4-dihydroxyphenylethylamine-1-C^{14}-HBr and 3-methoxy-4-hydroxybenzoic acid were synthesized in this laboratory. The 3-methoxy analogues of norepinephrine, epinephrine and dihydroxyphenylethylamine were supplied as the hydrochlorides by Dr. Bernard Witkop, National Institute of Arthritis and Metabolic Diseases.

Measurement of catecholamines in tissue and urine. Tissues were homogenized in 0.1 N HCl and 15 per cent trichloroacetic acid (1:2:2). The protein precipitate was removed by centrifugation and the supernatant solution extracted with water-saturated ether to remove the trichloroacetic acid. Catecholamines were extracted from acid-hydrolyzed urine by the method of von Euler and Orwen (2). The colorimetric method of von Euler and Hamberg (3) was used to measure epinephrine and norepinephrine in tissue extracts when the concentrations of the amines were adequate. Optical density was measured with a Beckman DU spectrophotometer. In some tumor extracts and in urine extracts, a modification (4) of the fluorometric procedure of Lund (5) was used, and the fluorescent product assayed using an Aminco-Bowman spectrophotofluorometer. The method of Weil-Malherbe and Bone (6) was used for determination of catecholamines in plasma. Although this method may not be as specific as other fluorometric procedures, it has a high order of sensitivity and when properly used is satisfactory in diagnosing pheochromocytoma.

Isolation and assay of methoxy analogues of catecholamines. Tumors were homogenized in a Waring blendor in six volumes of 0.01 N HCl. Portions of the homogenates were adjusted to pH 10.0 by addition of a saturated solution of sodium carbonate and extracted three times with five volumes of *n*-butanol. To the combined butanol extracts were added an equal volume of heptane and 0.2 volume of 0.5 N HCl. After shaking, the aqueous layer was evaporated to dryness *in vacuo* and the residue

* Part of the work presented here has been used by Lemuel C. Leeper in a thesis in partial fulfillment of the degree of Doctor of Philosophy, Department of Chemistry, Georgetown University, Washington, D. C., April, 1958.

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[1] They were hospitalized at the National Naval Medical Center, Bethesda, Md.; Georgetown University Hospital, Washington, D. C.; Walter Reed Army Medical Center, Washington, D. C.; and Southwest Foundation for Research and Education, San Antonio, Tex.

taken up in 10 ml. of methanol. The methanol was then evaporated under a stream of nitrogen to 0.4 ml. and portions were subjected to chromatography on Whatman No. 1 paper using two different solvents. The chromatograms were allowed to develop overnight in either isopropanol-5 per cent NH_3 (8:2), or butanol-acetic acid-water (8:1:1). After drying in air the chromatograms were sprayed with a freshly prepared solution of 2,6-dichloroquinone chloroimide (0.05 per cent w/v in ethanol) followed by borate buffer, pH 9.0 (7). Solutions of the authentic compounds in 0.01 N HCl and material eluted from the chromatograms were assayed fluorometrically (activation at 280 mμ and fluorescence at 330 mμ).

The enzyme which methylates the 3-hydroxy group of norepinephrine and its congeners is relatively nonspecific. In studies on *o*-methylation by tumor slices the conversion of 3,4-dihydroxybenzoic acid to 3-methoxy-4-hydroxybenzoic acid was utilized since the latter compound is more easily isolated and assayed than is the 3-methoxy metabolite of norepinephrine. 3-Methoxy-4-hydroxybenzoic acid was isolated from incubation mixtures after acidification (pH 2.0) and salt saturation by shaking twice with 20 ml. portions of ethyl acetate. The solvent phase was concentrated *in vacuo* to 0.3 ml. and transferred to Whatman No. 3 paper for chromatography. The chromatograms were developed with isopropanol-5 per cent NH_3 (8:2) and sprayed with the phenol reagent as described above. The 3-methoxy acid formed was compared with authentic compound (Rf 0.35).

Isolation and assay of radioactive norepinephrine. The procedure for isolation of norepinephrine-C^{14} from tumor slices and homogenates was the same as described previously for adrenal tissue (8). Extracts of urine prepared according to the procedure of von Euler and Orwen (2) were concentrated *in vacuo* and carried through the same procedure. Milligram-quantities of carrier norepinephrine were added to measured aliquots of the isolated norepinephrine-C^{14} and the catecholamine was then crystallized to constant specific activity and measured according to the procedure described by Udenfriend and Wyngaarden (4).

Enzymatic studies on tumor. All steps in preparing tissue slices and homogenates were carried out at 3° C. within two hours after removal of the tumors. Homogenates were prepared in a Potter homogenizer using isotonic KCl (one part tissue to two parts KCl). Tumor slices were prepared with a Stadie tissue slicer. Dopa decarboxylase activity was measured manometrically as described by Schales (9) and monoamine oxidase activity by a method described previously (10). The conditions used for incubation in studies with radioactive precursors of norepinephrine and in methylation of catechols by tumor slices are described in the appropriate tables.

TABLE I

Catecholamines in plasma, urine and tumor *

Case no.	Age	Sex	Time	Plasma NE†	Urinary NE+E‡	Tumor NE	Tumor E§	Tumor weight	Location (area)
			mo./yr.	*μg./L.*	*μg./24 hr.*	*mg./Gm.*	*mg./Gm.*	*Gm.*	
1	6	M	4/55	68.5		9.8	I	22	Right suprarenal
			Postop.	8.1					
			11/57	13.3	2,200	5.0	I	15	Neck
			Postop.		825				Site undetermined
2	34	F	6/55	60.8		8.0	1.7	160	Left suprarenal
3	8	F	9/55	65.6		4.0	I	36	Right suprarenal
			Postmortem			3.5	I	30	Left suprarenal
						1.8	I	20	Thorax
4	6	F	10/55	16.8		1.4	0.2	1.4	Right suprarenal
						0.2	I	1.7	Bifurcation of aorta
			12/55	9.9					
5	33	F	12/55	22.0					
			5/56		1,500	3.5	I	20	Right suprarenal
						1.8	I	22	Left suprarenal
			Postop.	5.7	190				
6	41	M	5/56		1,000	1.9	I	46	Right suprarenal
7	44	F	9/57		1,900	9.8	2.1	430	Right suprarenal

* Specimens of tumor were received in seven additional cases and in five of these, acidified urine specimens were also submitted. The tumors contained 0.5 to 7.1 mg. per Gm. norepinephrine; only one tumor contained a significant amount of epinephrine, 5.8 mg. per Gm. The values for epinephrine plus norepinephrine in urine were: 430, 820, 1,600, 1,600 and 3,000 μg. per day.

† Usual range is 3 to 6 μg. per L. norepinephrine and <1.0 μg. per L. epinephrine. Epinephrine values were within normal limits in all cases wherein plasma assays were done.

‡ Expressed as norepinephrine-equivalent. Usual range is 50 to 200 μg. per day.

§ I, insignificant amount, *i.e.* <5 per cent of norepinephrine value.

EXPERIMENTAL RESULTS

Since the clinical features of pheochromocytoma are well known, only the chemical findings in our cases will be presented.[2]

Measurement of catecholamines in plasma, urine and tumor

Since the original report by Engel and von Euler (12) of increased urinary excretion of norepinephrine in two patients with pheochromocytoma, the diagnostic value of chemical measurements of norepinephrine and epinephrine in plasma and urine has been demonstrated repeatedly. Large amounts of norepinephrine and often epinephrine also have been found in the tumors. Our experience is summarized in Table I. In this group of patients, the tumors contained norepinephrine as the predominant amine. This was true also of those located in the suprarenal areas.

Studies on biosynthesis of norepinephrine

The pathway for biosynthesis of norepinephrine in the body is now considered to be as shown in Figure 1. Although this metabolic sequence was first postulated in 1939 by Blaschko (13), convincing evidence could not be obtained even in experimental animals until the advent of isotopic tracer techniques and chromatographic methods for separating the three amines, 3,4-dihydroxyphenylethylamine (dopamine), norepinephrine and epinephrine. It has now been shown in animals that phenylalanine-C^{14} (which gives rise to tyrosine), tyrosine-C^{14}, 3,4-dihydroxyphenylalanine-C^{14} (dopa) and dopamine-C^{14} are precursors of adrenal norepinephrine and epinephrine. It is usually assumed that the formation of norepinephrine precedes that of epinephrine. Langemann (14) and Burger and Langemann (15) have reported that chromaffin tissue is rich in dopa decarboxylase. Little is known of the other cellular catalysts in the biosynthetic pathway.

A. In vitro. Portions of tumor-tissue from the patients in Cases 2, 5 and 6 were fortified with all the probable cofactors and incubated with radioactive dopa and dopamine. As shown in Table II, significant radioactivity appeared in norepinephrine isolated from the incubation mixtures in all experiments. Measurement of dopa decarboxylase activity in two tumors (Cases 5 and 6) gave values of 8,360 and 2,500 μg. dopa metabolized per Gm. per hour. This is a very high order of activity and is comparable to that found in guinea pig kidney, an extremely rich source of the enzyme.

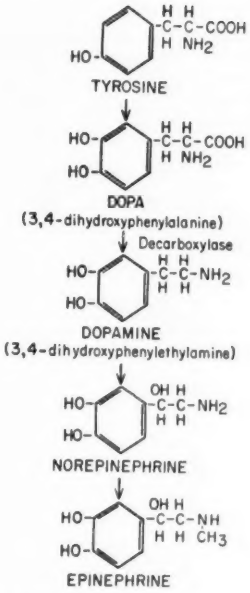

FIG. 1. THE CATECHOLAMINE PATHWAY OF TYROSINE METABOLISM

TABLE II

Formation of norepinephrine from C^{14}-labeled precursors by pheochromocytoma in vitro

Case no.	C^{14} substrate*	Radioactivity of substrate	Activity in isolated norepinephrine	
		cpm	*cpm/μM*	*total cpm*
2a†	Dopa	2,500,000	3,300	21,100
5a	Dopamine	800,000	652	4,300
6a	Dopamine	800,000	1,367	10,400
6b	Dopamine	800,000	840	6,400

* C^{14} compound incubated with 667 mg. tumor in air for one and one-half to three hours. In addition to substrate, the incubation mixtures contained 1 μM diphosphopyridine nucleotide (DPN), 10 μM nicotinamide, 2 μM adenosine triphosphate (ATP), 250 μM $MgCl_2$, 0.2 ml. 0.5 M phosphate buffer pH 7.0, and water to a total volume of 3.0 ml.

† a, homogenates; b, slices.

[2] Cases No. 1, 3, 4 and 5 (see Table I above) have been reported by Cone, Allen and Pearson (11).

TABLE III

*Urinary excretion of norepinephrine-C^{14} after administration of dopamine-C^{14} **

Collection periods	Activity in norepinephrine		
	Case 1	Case 5	Case 6
hrs.	*cpm/μM*	*cpm/μM*	*cpm/μM*
24		2,960	2,450
48		185	370
72		80	120
96			trace
4	9,440		
8	2,220		
12	1,000		
16	785		
24	525		

* 5×10^6 cpm administered intravenously.

B. In vivo. Additional proof of the precursor relationship of dopa and dopamine to norepinephrine in man was obtained by studies on four patients (Cases 1, 4, 5 and 6). The radioactive precursors were dissolved in 20 ml. of physiological NaCl solution and administered intravenously to these patients over a period of 10 minutes. In Case 4 a significant amount of radioactivity was found in norepinephrine isolated from urine collected during the first 24 hours after administration of 5 μc. of dopa-C^{14} (1.2 mg.) but quantitative studies were not carried out. The results of studies in three patients given 5 μc. of dopamine-C^{14} (1.2 mg.) are shown in Table III. In the studies on Patients No. 5 and 6, 24 hour collections of urine were made since a relatively slow rate of norepinephrine turnover analogous to that previously found in animal adrenals (4) was anticipated. Although large amounts of radioactivity were incorporated into the norepinephrine, the specific activity fell at such a rapid rate as to make estimates of turnover difficult using 24 hour collection periods. In a subsequent study in Case 1, urine collections were obtained at four hour intervals in order to obtain more precise information on turnover rate. It can be seen that a high degree of labeling of norepinephrine occurred within the first four hours. The specific activity of the urinary norepinephrine declined precipitously during the first 12 hours with a slower rate of decline occurring thereafter. The changes in specific activity observed between 48 and 72 hours in the first two experiments and after 12 hours in the third experiment most likely are indicative of the turnover rate of norepinephrine in the tumors. With proper assumptions, a half-life value for tumor norepinephrine may be calculated which is of the order of 8 to 12 hours. The initial, very rapid turnover phase is not easily explained. It may signify norepinephrine of nonpheochromocytoma origin, such as sympathetic nerve. This possibility is currently being investigated in normal individuals. It may also represent norepinephrine made by the tumor which does not mix with the norepinephrine stored in the tumor. The amount of dopamine administered in these experiments did not result in any marked increase in the synthesis of norepinephrine since the daily urinary excretion of norepinephrine did not change significantly during the experiments.

Total urinary C^{14} excretion following administration of dopamine-C^{14} was measured in one experiment (Case 6). Seventy-five per cent of the radioactivity was excreted in the first 24 hours, four per cent in the second 24 hours and less than one per cent in two subsequent days.

Although norepinephrine can be converted to epinephrine, the process was not found to be reversible in the calf adrenal gland (8). Since it has been demonstrated that a system for the demethylation of certain N-methyl amines does exist in the animal tissues (16), the following experiment was designed to investigate the possible conversion of epinephrine to norepinephrine in man. Five μc. (2.78 mg.) of radioactive epinephrine were infused intravenously over a period of several hours in Patient No. 7 and 24 hour urine collections were obtained starting at the beginning of the infusion. The norepinephrine from the first day's collection was isolated and recrystallized and its radioactivity measured. The results obtained are shown in

TABLE IV

Comparison of epinephrine and dopamine as precursors of norepinephrine in vivo

C^{14} compound	Total counts administered	Activity in isolated norepinephrine*
		cpm/μM
Epinephrine	5,000,000	<24
Dopamine†	5,000,000	2,580

* First 24 hour urine.
† Average of three experiments cited in Table III.

Table IV; a comparison is made with the findings following dopamine-C^{14} adminstration. It is obvious that there was little if any demethylation of epinephrine to form norepinephrine.

Studies on the metabolism of norepinephrine

Little was known of the fate of norepinephrine and epinephrine in the body until recently. The studies of von Euler and Luft (17) and Goldenberg, Serlin, Edwards and Rapport (18) showed clearly that only a small percentage of norepinephrine given intravenously is excreted in the urine. In 1957, Armstrong, McMillan and Shaw (19) reported the isolation of 3-methoxy-4-hydroxymandelic acid from human urine and showed that it was a major product of norepinephrine metabolism. Axelrod (20) then showed that the 3-hydroxy position of norepinephrine can be methylated by animal tissues; he also presented evidence (21) that *o*-methylation followed by oxidative deamination is the major pathway of metabolism in rats. Studies in this laboratory (22) using a partially purified preparation of monoamine oxidase have shown conversion of norepinephrine to 3,4-dihydroxymandelic acid and *o*-methylnorepinephrine (normetanephrine) to 3-methoxy-4-hydroxymandelic acid. *o*-Methylation of many catechol compounds has been demonstrated (20, 22) *in vitro*, including 3,4-dihydroxymandelic acid.

From this work one would surmise that the major portion of norepinephrine in patients with

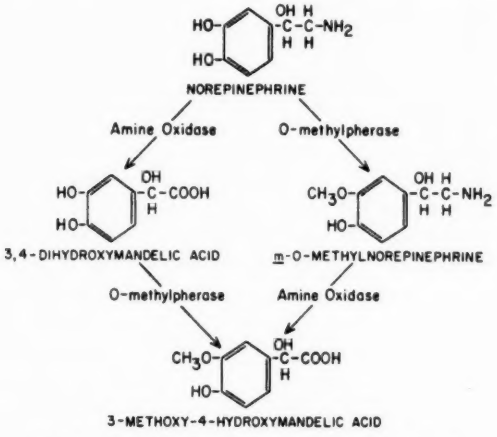

FIG. 2. THE MAJOR ROUTES OF METABOLISM OF NOREPINEPHRINE

TABLE V

Chromatographic behavior of methoxy analogues of catecholamines and the compound extracted from four pheochromocytomas

Compound	Rf: Isopropanol-5% NH_3 (8:2)	Rf: Butanol-acetic acid-water (8:1:1)	Color with phenol reagent
1	0.62–0.66	0.31	Blue
NMN*	0.65	0.30	Blue
ME*	0.80	0.28	Blue
MD*	0.77	0.40	Tan-pink

* Abbreviations correspond to the 3-methoxy analogues of norepinephrine (NMN), epinephrine (ME), and 3,4-dihydroxyphenylethylamine (MD).

pheochromocytoma is metabolized by the pathways shown in Figure 2. It is of considerable importance to determine which pathway predominates and which, if either, may limit the physiologic actions of norepinephrine. The following studies were designed to answer some of these questions.

A. In vitro. A preliminary report of some of these findings has been presented elsewhere (23). Portions of four tumors were studied for the presence of methoxy-substituted catecholamines as described in the section on methods. The results of these studies, summarized in Table V, indicate that all the tumors contained a butanol-extractable base identical with normetanephrine (NMN) as shown by its Rf values and color obtained with the phenol reagent and ninhydrin. In several instances the areas corresponding to NMN on the chromatograms obtained with isopropanol-NH_3 were eluted and rechromatographed with butanol-acetic acid. The Rf values obtained were again identical with those of authentic NMN. The tumor in Case 7, which contained epinephrine as well as norepinephrine, was found to contain two additional phenolic substances, Compounds 2 and 3. Compound 2 appeared on the chromatograms between NMN and the methoxy analogue of epinephrine (ME) and gave a bluish-green color with the phenol reagent, unlike that of the three available standards. Compound 3 exhibited Rf values and color (tan-pink) identical with those of the methoxy analogue of dopamine (MD).

The areas on the chromatogram corresponding to Compounds 1, 2 and 3 were eluted with 0.01

N HCl and their fluorescence characteristics were compared with authentic NMN, ME and MD, using the Aminco-Bowman spectrophotofluorometer. All three componds and the reference standards exhibited maximal activation at 280 mμ and maximal fluorescence at 330 mμ. Further evidence for the identity of Compound 1 with NMN was obtained by eluting it from the chromatogram and subjecting it to distribution between equal volumes of 0.5 M borate buffer pH 10, and *n*-butanol. Analyses were carried out spectrophotofluorometrically. The distribution coefficients (concentration in butanol/concentration in aqueous) obtained for Compound 1 and NMN were 0.43 and 0.44, respectively, whereas values for ME and MD were 4.0 and 6.1. One tumor (Case 7), subjected to quantitative assay for NMN using a combination of paper chromatography and spectrophotofluorometry, was found to contain about 25 μg. per Gm.

Further studies revealed that the tumor (Case 7) is capable of converting catechols to their 3-methoxy analogues. However, the tissue could do this only when presented with the activated form of methionine, S-adenosylmethionine (see Table VI). It is not known whether the tumor can synthesize S-adenosylmethionine *in vivo*. We have also found that many organ tissues which normally contain norepinephrine such as heart, brain and adrenal gland are capable of methylating catechol compounds under these circumstances. The same tumor (Case 7) was also found to have considerable monoamine oxidase activity with 600 μg. serotonin being metabolized per Gm. per hour.

TABLE VI

Assay for o-methylating enzyme in pheochromocytoma

Experiment no.	Incubation system*	Results†
1	Slices only	0
2	Slices + 500 μg. substrate‡	0
3	Slices + 500 μg. substrate + 1 μM ATP + 250 μM Mg + 6.5 μM methionine	0
4	Slices + 500 μg. substrate + 2 μM S-adenosylmethionine + 250 μM Mg	+

* Incubation was for three hours in air at 37° C. In addition to the components listed, each beaker contained 0.3 ml. of 0.5 M phosphate buffer, pH 7.7, and water to make a final volume of 3.0 ml. The tissue slices weighed 500 mg. in each instance.

† The *o*-methyl acid was extracted from the incubation mixture with ethyl acetate after acidification and salt saturation. The extract was then evaporated to small volume (0.5 ml.) and chromatographed for *o*-methyl compounds as described under "Methods."

‡ Substrate was 3,4-dihydroxybenzoic acid.

B. In vivo. While the measurement of 3-methoxy-4-hydroxymandelic acid is a useful means of diagnosing pheochromocytoma (19), another possible approach is revealed by the findings of LaBrosse, Axelrod and Sjoerdsma (24) in two of our patients. Making quantitative use of paper chromatography, it was found that Patients 1 and 7 were excreting about 20 mg. of 3-methoxy-4-hydroxymandelic acid per day but also 10 to 30 mg. of normetanephrine when the norepinephrine excretion was approximately 2 mg. per day.

DISCUSSION

It would appear from these studies that the pathway for biosynthesis of norepinephrine in pheochromocytoma is the same as that shown previously in animal adrenal gland. The chemical disorder in patients with this tumor may, therefore, be thought of as a disturbance in the metabolism of tyrosine. Preliminary studies suggest that a lower fasting plasma tyrosine level may exist in these patients than in normals (25).

Two of the reactions in the biosynthesis of norepinephrine which were demonstrated here were the decarboxylation of dopa and the side chain hydroxylation of dopamine. Other studies in this laboratory have shown that pheochromocytoma is more active in hydroxylating dopamine than are various animal tissues, including adrenal gland (26). Recent reports by Weil-Malherbe (27) and McMillan (28) indicate that some pheochromocytomas may produce dopamine as the predominant catecholamine. It is possible that there are tumors which produce only dopamine because of an inability to convert it to norepinephrine. Since dopamine is a relatively weak pressor agent, such a tumor might erroneously be termed "nonfunctioning." Furthermore, a dopamine producing tumor may not be detected by some of the bioassay and chemical procedures which are carried out on blood and urine. A simple chemical assay on the tumor itself would reveal this, however. In this respect it is now advisable and feasible to carry out chemical analyses of many secreting tumors which are still being classified only by histological methods.

The finding of the methoxy analogue of norepinephrine (normetanephrine) in the tumors is the first demonstration of this amine in human tissues. Although procedures for measuring urinary metabolites of norepinephrine are still only semiquantitative, it would appear from studies in pheochromocytoma that the two major metabolites in man are normetanephrine and 3-methoxy-4-hydroxymandelic acid. Development of satisfactory methods for measuring these substances will have many applications, including alternate means for diagnosing pheochromocytoma.

The rapid turnover rate of norepinephrine in the three patients with pheochromocytoma (half-life of about 10 hours) is in sharp contrast to the relatively slow turnover in animal (and presumably human) adrenal gland (half-life of several days) (4). The patients (Cases 1, 5 and 6) studied had tumors which contained only norepinephrine, indicating probable neural origin. This may signify similar rapid turnover of the amine in sympathetic nerves. Considering the fact that only a small percentage of norepinephrine entering the blood is excreted in the urine (17, 18), it can be concluded from the amounts of norepinephrine in the urines and tumors in Cases 5 and 6 that release of an amount equivalent to the entire tumor pool over a period of 24 hours would be required to produce the amount of urinary norepinephrine found daily in each case. This is entirely consistent with the experimentally determined turnover rates for tumor norepinephrine. On the other hand, it is obvious that Patient No. 7 could not have survived if the entire tumor content of norepinephrine (approximately 4,200 mg.) were released into the blood every day. The urinary excretion of catecholamines in this case was only slightly greater than in the previous cases. A slower rate of turnover must be postulated in this instance. It may be more than coincidence that the tumor in Case 7 was the largest (430 Gm.) in this series and also contained epinephrine. The other relatively large tumor (Case 2, 160 Gm.) also contained a considerable amount of epinephrine. The same correlation between size of tumor and presence of epinephrine can be seen in the data of von Euler and Ström (29). We would like to speculate that these large tumors originate from the adrenal gland and retain its properties of storage, slow turnover and ability to produce epinephrine. This may account for their growth to a much larger size before the amounts of catecholamines which they secrete are sufficient to produce clinical manifestations.

SUMMARY

1. Chemical studies on seven patients with pheochromocytoma are presented.

2. Studies with C^{14}-labeled precursors *in vitro* and *in vivo* corroborate present concepts of the biogenesis of norepinephrine from dihydroxyphenylalanine and dihydroxyphenylethylamine. Enzymes involved in the biogenesis and metabolism of norepinephrine were demonstrated in the tumors.

3. The 3-methoxy analogue of norepinephrine (normetanephrine) was found in each of four pheochromocytomas studied. This is the first demonstration of this amine in a human tissue.

4. Preliminary evidence is cited which indicates that the two major urinary metabolites of norepinephrine in patients with this tumor are normetanephrine and 3-methoxy-4-hydroxymandelic acid. Measurements of these compounds may afford an alternate means of diagnosis.

5. Estimations of norepinephrine turnover rate in three patients suggest a relatively short half-life in some pheochromocytomas as compared to animal adrenal glands.

6. Some implications of these findings are discussed.

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REFERENCES

1. von Euler, U. S. Noradrenaline; Chemistry, Physiology, Pharmacology, and Clinical Aspects. Springfield, Ill., Charles C Thomas, 1956.
2. von Euler, U. S., and Orwen, I. Preparation of extracts of urine and organs for estimation of free and conjugated noradrenaline and adrenaline. Acta physiol. scand. 1955, **33**, Suppl. 118, 1.

3. von Euler, U. S., and Hamberg, U. Colorimetric estimation of noradrenaline in the presence of adrenaline. Science 1949, **110**, 561.
4. Udenfriend, S., and Wyngaarden, J. B. Precursors of adrenal epinephrine and norepinephrine *in vivo*. Biochim. biophys. Acta 1956, **20**, 48.
5. Lund, A. Simultaneous fluorimetric determinations of adrenaline and noradrenaline in blood. Acta pharmacol. (Kbh.) 1950, **6**, 137.
6. Weil-Malherbe, H., and Bone, A. D. The adrenergic amines of human blood. Lancet 1953, **1**, 974 .
7. Bray, H. G., and Thorpe, W. V. Analysis of phenolic compounds of interest in metabolism *in* Methods of Biochemical Analysis, David Glick, Ed. New York, Interscience Publishers, Inc., 1954, vol. I, p. 27.
8. Rosenfeld, G., Leeper, L. C., and Udenfriend, S. Biosynthesis of norepinephrine and epinephrine by the isolated perfused calf adrenal. Arch. Biochem. 1958, **74**, 252.
9. Schales, O. Amino acid decarboxylases of animals *in* Methods in Enzymology, Sidney P. Colowick and Nathan O. Kaplan, Eds. New York, Academic Press Inc., 1955, vol. II, p. 195.
10. Sjoerdsma, A., Smith, T. E., Stevenson, T. D., and Udenfriend, S. Metabolism of 5-hydroxytryptamine (serotonin) by monoamine oxidase. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 36.
11. Cone, T. E., Jr., Allen, M. S., and Pearson, H. A. Pheochromocytoma in children. Report of three familial cases in two unrelated families. Pediatrics 1957, **19**, 44.
12. Engel, A., and von Euler, U. S. Diagnostic value of increased urinary output of noradrenaline and adrenaline in phaeochromocytoma. Lancet 1950, **2**, 387.
13. Blaschko, H. The specific action of *l*-dopa decarboxylase. J. Physiol. (Lond.) 1939, **96**, 50 *P*.
14. Langemann, H. Enzymes and their substrates in the adrenal gland of the ox. Brit. J. Pharmacol. 1951, **6**, 318.
15. Burger, M., and Langemann, H. Bestimmungen von Adrenalin und Noradrenalin sowie von Decarboxylase- und Aminoxydase-Aktivitäten in Zellfraktionen von Phäochromocytomen. Klin. Wschr. 1956, **34**, 941.
16. Axelrod, J. The enzymatic demethylation of ephedrine. J. Pharmacol. exp. Ther. 1955, **114**, 430.
17. von Euler, U. S., and Luft, R. Noradrenaline output in urine after infusion in man. Brit. J. Pharmacol. 1951, **6**, 286.
18. Goldenberg, M., Serlin, I., Edwards, T., and Rapport, M. M. Chemical screening methods for the diagnosis of pheochromocytoma. Amer. J. Med. 1954, **16**, 310.
19. Armstrong, M. D., McMillan, A., and Shaw, K. N. F. 3-Methoxy-4-hydroxy-D-mandelic acid, a urinary metabolite of norepinephrine. Biochim biophys. Acta 1957, **25**, 422.
20. Axelrod, J. O-Methylation of epinephrine and other catechols in vitro and in vivo. Science 1957, **126**, 400.
21. Axelrod, J., Inscoe, J. K., Senoh, S., and Witkop, B. O-Methylation, the principal pathway for the metabolism of epinephrine and norepinephrine in the rat. Biochim biophys. Acta 1958, **27**, 210.
22. Leeper, L. C., Weissbach, H., and Udenfriend, S. Studies on the metabolism of norepinephrine, epinephrine and their *o*-methyl analogues by partially purified enzyme preparations. Arch. Biochem. 1958, **77**, 417.
23. Sjoerdsma, A., King, W. M., Leeper, L. C., and Udenfriend, S. Demonstration of the 3-methoxy-analog of norepinephrine in man. Science 1958, **127**, 876.
24. LaBrosse, E. H., Axelrod, J., and Sjoerdsma, A. Urinary excretion of normetanephrine by man. Fed. Proc. 1958, **17**, 386.
25. Waalkes, T. P., and Udenfriend, S. A fluorometric method for the estimation of tyrosine in plasma and tissues. J. Lab. clin. Med. 1957, **50**, 733.
26. Leeper, L. C. Studies on the biosynthesis and inactivation of norepinephrine and epinephrine. Ph.D. Thesis, Department of Chemistry, Georgetown University, Washington, D. C., 1958.
27. Weil-Malherbe, H. Phaeochromocytoma, catechols in urine and tumour tissue. Lancet 1956, **1**, 282.
28. McMillan, M. Identification of hydroxytyramine in a chromaffin tumor. Lancet 1956, **2**, 284.
29. von Euler, U. S., and Ström, G. Present status of diagnosis and treatment of pheochromocytoma. Circulation 1957, **15**, 5.

ON THE MECHANISM OF ACIDOSIS IN CHRONIC RENAL DISEASE *

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It has been pointed out (1) that, in terms of modern acid-base chemistry, the defects reponsible for the acidosis of renal disease could be: *1*) impaired renal excretion of acid in the form of ammonium and titratable acidity, *2*) loss of bicarbonate (alkali) in the urine due to a failure of renal bicarbonate conservation or *3*) a combination of these two defects.

The two main components of urine responsible for the removal of hydrogen [1] from the body are ammonium and titratable acid. Under the stress of endogenous or exogenous acidosis the normal kidney responds by increasing the excretion of both these moieties, but the increment in ammonium usually far exceeds that in titratable acid. It has long been known that the basal excretion of ammonium is reduced in patients with renal disease, and that the capacity to increase ammonium excretion in response to acidosis is also impaired (2–7). Somewhat less attention has been directed towards the ability of the diseased kidney to excrete titratable acid, although it was demonstrated many years ago that patients with uremic acidosis can elaborate very acid urines (2, 3). In so-called "renal tubular acidosis" it is generally accepted that characteristically this function is lost and that all urines are relatively alkaline (8).

Whether there is any disturbance in bicarbonate reabsorption in renal disease is not clear from available data. With one exception (9) there has been no report of such a defect in patients with uremic acidosis. A recent study of the tubular response to acute bicarbonate loading in such patients has, in fact, led to the conclusion that bicarbonate reabsorption is normal (10). On the other hand, defects in bicarbonate reabsorption have been frequently identified in patients with so-called "renal tubular acidosis" and the Fanconi syndrome (11–13).

No previous attempt has been made to study the sequence of events which occur as patients with renal insufficiency spontaneously become acidotic. The present study has been undertaken in an effort to obtain this information by means of balance studies in patients whose acidosis was initially corrected by administration of alkali. The data to be presented provide some indication of the relative importance of the various renal defects and of their functional relationships. These experiments also appear to throw some light on the mechanisms which allow patients with renal disease to achieve a new steady state of hydrogen ion balance at subnormal plasma bicarbonate concentrations.

METHODS

Balance studies were carried out on five patients. Four of the five (P.D., D.H., H.G. and R.M.) appeared to have chronic glomerulonephritis according to the usual clinical and laboratory criteria; three of these four also had a history of urinary tract infection at some time prior to their study and may also have had chronic pyelonephritis. The fifth patient had a well-documented history of renal tubular acidosis, nephrolithiasis and recurrent urinary tract infections. (For purposes of clarity, this pa-

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‡ Research Fellow of the Arthritis and Rheumatism Foundation.

§ Public Health Service Research Fellow of the National Heart Institute.

[1] The term "hydrogen" as used in this discussion is not restricted to that very small quantity of hydrogen which exists in the free ionized form, but is meant to include hydrogen which has been bound to body buffers.

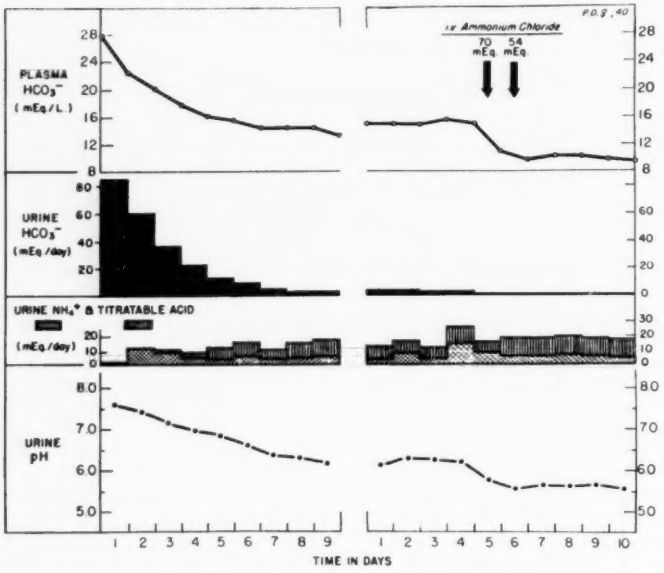

FIG. 1. PLASMA BICARBONATE CONCENTRATION, URINE pH AND DAILY EXCRETION OF BICARBONATE, AMMONIUM AND TITRATABLE ACID IN PATIENT P.D.

Observations began the day after acidosis had been corrected and treatment with alkali had been stopped. The break in the middle of the observations indicates an interruption of seven days. See text for details.

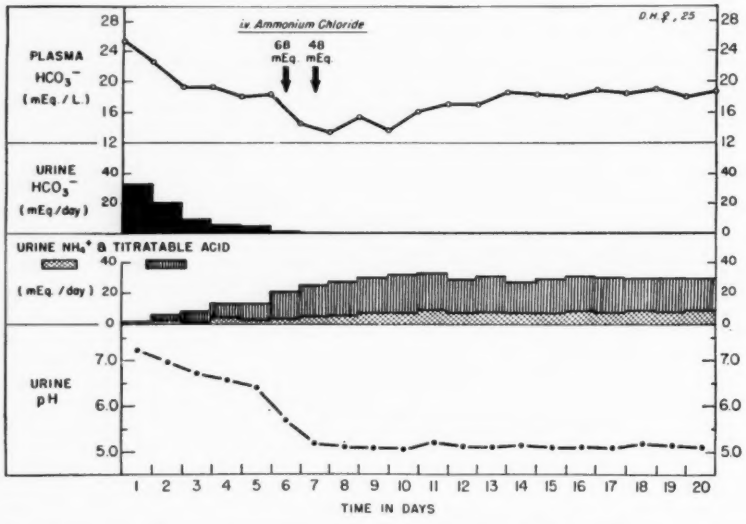

FIG. 2. PLASMA BICARBONATE CONCENTRATION, URINE pH AND DAILY EXCRETION OF BICARBONATE, AMMONIUM AND TITRATABLE ACID IN PATIENT D.H.

Observations began the day after acidosis had been corrected and treatment with alkali had been stopped.

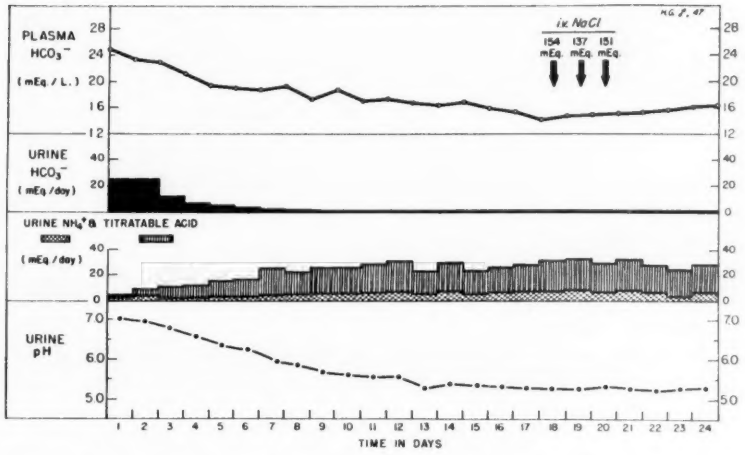

FIG. 3. PLASMA BICARBONATE CONCENTRATION, URINE pH AND DAILY EXCRETION OF BICARBONATE, AMMONIUM AND TITRATABLE ACID IN PATIENT H.G.

Observations began the day after acidosis had been corrected and treatment with alkali had been stopped.

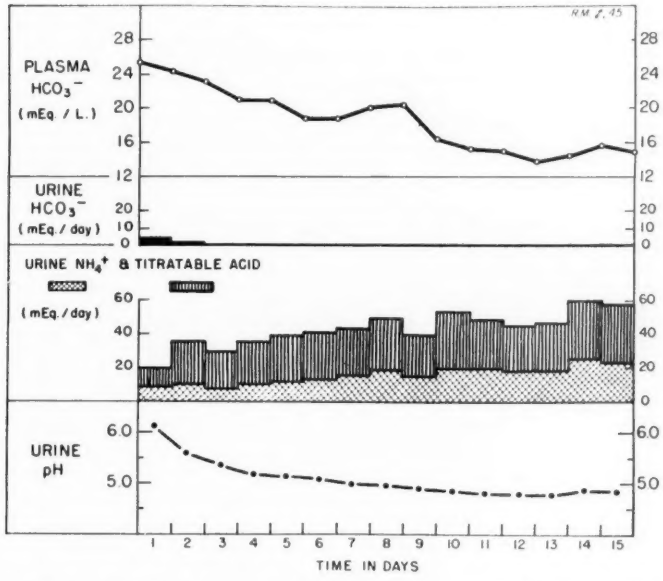

FIG. 4. PLASMA BICARBONATE CONCENTRATION, URINE pH AND DAILY EXCRETION OF BICARBONATE, AMMONIUM AND TITRATABLE ACID IN PATIENT R.M.

Observations began the day after acidosis had been corrected and treatment with alkali had been stopped.

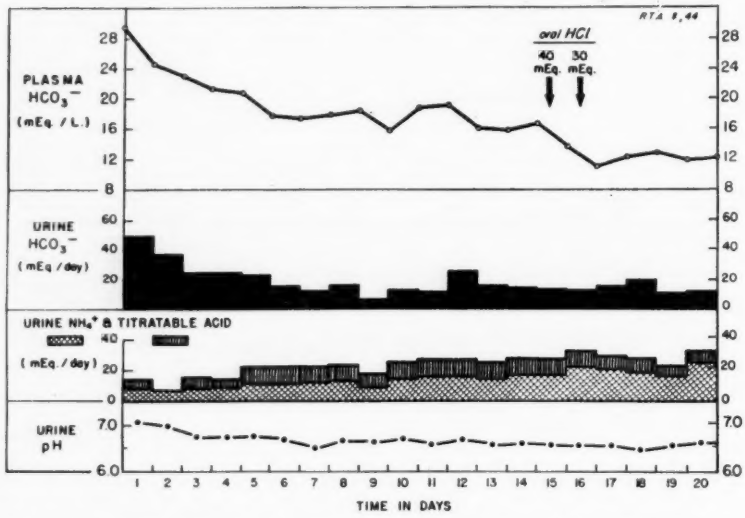

FIG. 5. PLASMA BICARBONATE CONCENTRATION, URINE pH AND DAILY EXCRETION OF BICARBONATE, AMMONIUM AND TITRATABLE ACID IN PATIENT R.T.A.

Observations began the day after acidosis had been corrected and treatment with alkali had been stopped.

TABLE I

Electrolyte balances: Patient P. D.

Day	Body wgt.	Intake					Urine									
		H_2O	Na	K	Cl	N	Vol.	pH	HCO_3	T.A.	NH_4	Na	K	Cl	P	N
	Kg.	*ml./day*	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*	*ml.*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mM/day*	*Gm./day*
							First study									
1	64.78	2,000	9	50	13	8.2	2,170	7.56	85	0	2	138	53	27	20	7.4
2	64.23	2,000	9	50	13	8.2	2,568	7.45	60	0	12	112	63	26	23	8.7
3	63.71	2,000	9	50	13	8.2	2,247	7.15	36	2	9	68	62	17	21	8.1
4	63.26	2,000	9	50	13	8.2	2,206	7.00	22	4	5	46	60	12	20	8.2
5	63.00	2,000	9	50	13	8.2	2,213	6.80	13	8	4	40	55	14	20	8.2
6	62.73	2,000	9	50	13	8.2	2,267	6.60	9	10	6	35	55	13	22	8.8
7	62.21	2,000	9	50	13	8.2	2,074	6.35	5	7	4	29	51	13	20	8.7
8	62.00	2,000	9	50	13	8.2	1,870	6.30	3	11	5	24	47	11	18	8.2
9	62.03	2,000	9	50	13	8.2	2,248	6.25	3	12	6	30	52	15	19	9.2
10	61.57															
							Second study									
1	61.29	2,000	9	50	13	8.2	1,745	6.10	4	9	4	49	24	37	10	5.4
2	61.38	2,000	9	50	13	8.2	2,075	6.25	4	9	8	43	28	31	11	5.8
3	61.11	2,000	9	50	13	8.2	2,097	6.25	3	8	4	38	30	27	11	5.7
4	60.87	2,000	9	50	13	8.2	2,140	6.20	3	11	15	36	33	25	13	6.0
5*	60.78	2,000	9	50	83	9.2	2,135	5.75	1	8	8	34	37	31	12	6.7
6†	60.38	2,000	9	50	67	9.0	1,683	5.55	1	13	6	23	34	26	10	5.6
7	60.60	2,000	9	50	13	8.2	2,001	5.60	1	13	6	27	38	31	12	6.2
8	60.47	2,000	9	50	13	8.2	2,123	5.60	1	13	7	30	39	33	12	6.6
9	60.17	2,000	9	50	13	8.2	1,788	5.60	1	13	6	23	36	25	12	6.1
10	60.03	2,000	9	50	13	8.2	1,825	5.55	1	13	5	25	35	26	13	6.4
11	59.63															

* 70 mm. NH_4Cl administered intravenously.
† 54 mm. NH_4Cl administered intravenously.

TABLE I—*Continued*

Day	Body wgt.	Stool				Plasma						
		Na	K	Cl	N	pH	HCO_3	Na	K	Cl	P	Creat.
	Kg.	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*
						First study						
1	64.78	1	12	2	1.1	7.46	27.9	143		95		10.8
2	64.23	1	12	2	1.1	7.33	22.6	140	5.2	97		
3	63.71	1	12	2	1.1	7.32	20.3	136	5.1	97		
4	63.26	1	12	2	1.1	7.30	17.9	138	5.2	97		
5	63.00	1	12	2	1.1	7.29	16.3	135	5.1	98		
6	62.73	1	12	2	1.1	7.29	15.7	135	4.8	97		
7	62.21	1	12	2	1.1	7.22	14.6	140	4.6	98		9.2
8	62.00	1	12	2	1.1	7.29	14.6	133	4.7	96	12.0	
9	62.03	1	12	2	1.1	7.25	14.7	143	4.9	92		
10	61.57					7.23	13.3	133	4.7	100		
						Second study						
1	61.29	1	10	2	1.1	7.25	15.0	139	3.7	106	7.8	8.3
2	61.38	1	10	2	1.1	7.26	15.0	142	4.3	105		
3	61.11	1	10	2	1.1	7.29	14.9	137	4.0	103	7.1	
4	60.87	1	10	2	1.1	7.28	15.6	141	4.3	102		
5*	60.78	1	10	2	1.1	7.26	15.1	138	4.4	103	5.7	
6†	60.38	1	10	2	1.1	7.20	11.0	140	4.7	108		
7	60.60	1	10	2	1.1	7.15	9.8	138	5.3	110	8.4	9.5
8	60.47	1	10	2	1.1	7.16	10.1	133	5.0	110		
9	60.17	1	10	2	1.1	7.19	10.1	135	5.0	111		
10	60.03	1	10	2	1.1	7.17	9.7	131	5.3			
11	59.63					7.16	9.4	131	4.6	103	8.9	

tient will be referred to as R.T.A.) Prior to study, all patients demonstrated a persistently low plasma bicarbonate concentration, ranging between 13 and 18 mEq. per L. At the time of study none of the patients had active infection except R.T.A. She was continuously maintained on 1 Gm. of chloramphenicol during the experimental period and usually had bacterial counts of less than 1,000 colonies per ml. in clean voided urine specimens.

Plan of study. Before starting the balance study, plasma bicarbonate was raised to between 25 and 29 mEq. per L. over a two to three day period by the administration of sodium bicarbonate intravenously and by mouth. Bicarbonate administration was then stopped and the patient placed on a constant weighed diet. In four experiments the sodium content of the diet was low (7 to 12 mEq. per day), and in the fifth (R.M.) it was normal.

TABLE II

Electrolyte balances: Patient R. M.

Day	Body wgt.	Intake					Urine									
		H_2O	Na	K	Cl	N	Vol.	pH	HCO_3	T.A.	NH_4	Na	K	Cl	P	N
	Kg.	*ml./day*	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*	*ml.*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mM/day*	*Gm./day*
1		2,480	52	65	55	14	2,840	6.10	4	11	9	191	64	122	22	15.9
2	72.32	2,480	52	65	55	14	2,140	5.60	1	26	10	115	67	69	19	13.1
3	72.13	1,980	52	65	55	14	1,680	5.35	1	22	8	60	61	47	16	10.6
4	72.39	2,480	52	65	55	14	1,960	5.15	1	25	11	71	75	45	20	12.7
5	72.58	2,480	52	65	55	14	2,150	5.10	1	28	12	71	80	47	21	13.3
6	72.36	2,480	52	65	55	14	2,180	5.05	1	28	14	65	72	41	20	12.6
7	72.27	2,480	52	65	55	14	2,530	4.95	1	28	16	68	78	48	23	14.4
8	71.88	2,480	52	65	55	14	2,520	4.95	1	30	19	62	76	43	24	13.9
9	71.69	2,480	52	65	55	14	1,820	4.90	1	24	16	41	58	31	17	10.6
10	71.20	2,480	52	65	55	14	2,650	4.85	1	34	20	56	74	45	23	14.0
11	70.99	2,480	86	65	89	14	2,195	4.80	1	29	20	42	70	35	20	12.7
12	71.13	2,480	153	65	156	14	2,088	4.80	1	27	19	59	61	52	19	11.9
13	71.46	2,680	153	65	156	14	2,188	4.80	1	28	19	77	61	70	18	12.5
14	71.63	2,480	153	65	156	14	2,840	4.85	1	35	25	127	81	116	25	17.6
15	71.43	2,480	149*	63*	137*	14	2,580	4.85	1	34	24	134	67	121	20	15.5
16	70.53															

* Intake figures corrected for rejected diet.

TABLE II—*Continued*

Day	Body wgt.	Stool				Plasma						
		Na	K	Cl	N	pH	HCO_3	Na	K	Cl	P	Creat.
	Kg.	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*
1		3	9	4	1.1	7.51	25.7	146	4.4			
2	72.32	3	9	4	1.1	7.40	24.3	146	4.5	99	8.2	
3	72.13	3	9	4	1.1	7.31	23.1	144	4.4	96	8.8	
4	72.39	3	9	4	1.1	7.33	21.2	140	4.6	96	9.4	
5	72.58	3	9	4	1.1	7.33	20.8	137	4.3	96	9.5	9.8
6	72.36	3	9	4	1.1	7.32	18.9	145	4.4	97	9.9	
7	72.27	3	9	4	1.1	7.26	18.8	137	4.2	97	9.8	
8	71.88	3	9	4	1.1	7.32	20.0	136	4.2	95	10.3	
9	71.69	3	9	4	1.1	7.31	20.5	136	4.2	96	10.4	
10	71.20	3	9	4	1.1	7.32	16.3	136	3.9	93	10.7	12.6
11	70.99	3	9	4	1.1	7.29	15.2	136	4.0	95	9.8	
12	71.13	3	9	4	1.1	7.28	14.9	139	4.0	97	9.9	
13	71.46	3	9	4	1.1	7.25	13.8	139	4.1	101	9.9	11.1
14	71.63	3	9	4	1.1	7.25	14.5	141	4.2	102	9.5	
15	71.43	3	9	4	1.1	7.29	15.7	140	4.2	103	8.4	
16	70.53	3	9	4	1.1	7.26	14.7	140	4.2	104	8.5	

In all other respects the diet was normal in composition. Balances were corrected for vomitus and rejected foods, which were always analyzed. In some instances ammonium chloride or hydrochloric acid was given for two days during the latter part of the study. In two patients supplementary salt was given for several days. Water intake was set at a level selected by the patients and maintained constant thereafter. Urines were placed under oil and refrigerated immediately after voiding. Saturated thymol and chloroform was used as a preservative.

The analytic procedures and the methods used in calculation of results, as well as other details of the balance technique, have been described in a previous paper (14).

RESULTS

Figures 1 to 5 show plasma bicarbonate concentration and the acid-base composition of the urine in all five patients. The complete balance data for Patients P.D., R.M. and R.T.A. are presented in Tables I to III.

Plasma composition

Bicarbonate and pH. During the first few days plasma bicarbonate concentration fell relatively rapidly in all patients to levels between 15 and 20 mEq. per L. In three (P.D., D.H. and R.T.A.) the plasma concentration appeared to reach a plateau within a week, while in two (H.G. and R.M.) there was a further slow decline of about 5 mEq. per L. over the next seven to 10 days.

Administration of HCl or NH_4Cl (P.D., D.H. and R.T.A.) over a two day period was followed by a fall in plasma bicarbonate of 5 to 6 mEq. per L. to final levels of between 10 and 13 mEq. per L. In two of the three patients (P.D. and R.T.A.) plasma bicarbonate concentrations remained at the new low level. In the third patient (D.H.) there was a rise of approximately 6 mEq. per L. over the next six days, restoring the plasma bicarbonate concentration to the level which had obtained prior to the administration of ammonium chloride. As shown in Tables I to III, plasma pH tended to fall as plasma bicarbonate concentration was reduced.

Sodium, potassium, chloride and phosphorus. Serum sodium, potassium, chloride and phosphorus concentrations showed only minor changes during the course of the study in four of the patients. In the fifth (R.T.A.), there was a gradual fall in sodium concentration from an initial value of 146 mEq. per L. to final levels of 124 mEq. per L., but chloride, potassium and phosphorus did not change significantly (Table III).

Plasma creatinine. Plasma creatinine concentration was elevated to between 8 to 13 mg. per cent in P.D., D.H., H.G. and R.M. There was no significant change during the course of each study. In R.T.A. plasma creatinine concentration was 1 mg. per cent at the beginning, and 0.9 mg. per cent at the end of the study.

Urine composition

Bicarbonate excretion. Four out of five subjects (Figures 1 to 3 and 5) showed significant urinary excretion of bicarbonate at a time when plasma bicarbonate levels were below 25 mEq.

per L. As plasma bicarbonate fell progressively, there was a stepwise reduction in bicarbonate excretion with the result that within five to seven days bicarbonate had virtually disappeared from the urine in all patients except R.T.A.

The cumulative bicarbonate loss during this phase of rapid excretion was approximately 260 mEq. in P.D., and approximately 75 mEq. in H.G. and D.H.

Patient R.T.A. continued to excrete significant amounts of bicarbonate in her urine (averaging 10 to 20 mEq. per day) throughout the entire study. There was no reduction in bicarbonate excretion following the administration of hydrochloric acid.

Urine pH. Urine pH fell progressively in all patients except R.T.A. as metabolic acidosis became more severe. Final pH values of 4.8 to 5.5 were attained in P.D., D.H., H.G. and R.M. The most abrupt fall in urine pH followed the administration of ammonium chloride to P.D. and D.H.

R.T.A., despite the administration of hydrochloric acid and a drop of plasma bicarbonate to 12 mEq. per L., achieved a minimal urine pH of only 6.5.

Titratable acid. In all patients excretion of titratable acid increased progressively during the initial five to 10 days of the study. The maximum value ranged between 11 to 34 mEq. per day with an average of 18 mEq. per day. In all patients except R.T.A., titratable acid accounted for approximately two-thirds of the total acid excretion throughout the period of study.

The administration of ammonium chloride produced a significant increase in titratable acid excretion in Patient D.H., with a rise from 11 to 29 mEq. per day as urine pH fell from 6.4 to 5.0. In P.D., a fall in urine pH from 6.2 to 5.6 was unaccompanied by a significant change in titratable acid. In R.T.A., whose urine pH was unaffected by acid administration, titratable acid excretion remained constant.

Ammonium excretion. All patients showed a marked limitation of ammonium excretion during the spontaneous development of acidosis, and following the administration of acid. The maximum ammonium excretions observed ranged from

TABLE III

Electrolyte balances: Patient R. T. A.

Day	Body wgt.	Intake					Urine									
		H_2O	Na	K	Cl	N	Vol.	pH	HCO_3	T. A.	NH_4	Na	K	Cl	P	N
	Kg.	*ml./day*	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*	*ml.*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mM/day*	*Gm./day*
1	42.82	5,720	7	47	10	6.2	5,765	7.05	50	6	9	108	31	37	17	6.9
2	42.15	5,720	7	47	10	6.2	5,530	6.95	38	0	8	87	37	33	18	7.7
3	41.54	5,720	7	47	10	6.2	5,605	6.75	25	7	9	57	35	25	15	6.7
4	41.05	5,720	7	47	10	6.2	5,375	6.70	25	5	10	41	46	18	18	7.0
5	41.03	5,720	7	47	10	6.2	5,500	6.75	24	11	12	33	49	15	21	7.2
6	40.69	5,720	7	47	10	6.2	4,960	6.70	16	11	12	24	51	14	21	6.9
7	40.83	5,720	7	47	10	6.2	4,940	6.50	13	10	13	16	51	11	18	6.9
8	40.93	5,720	7	47	10	6.2	5,720	6.65	17	10	14	17	55	11	21	7.7
9*	40.33	3,245	6†	32†	9†	5.2†	2,308	6.60	7	8	10	9	38	7	15	5.3
10	40.00	4,940	7	47	10	6.2	4,300	6.70	13	11	15	11	51	9	20	7.7
11	40.14	5,720	7	47	10	6.2	5,000	6.55	12	11	16	12	51	8	20	7.5
12	40.35	5,720	7	47	10	6.2	5,600	6.70	26	11	16	17	52	9	19	7.6
13	39.90	5,720	7	47	10	6.2	4,970	6.60	16	11	15	11	50	7	17	7.0
14	40.02	5,720	7	47	10	6.2	5,260	6.60	15	11	17	11	51	6	18	7.4
15‡	39.90	5,720	7	47	50	6.2	5,350	6.60	14	11	17	11	53	7	20	7.5
16§	39.51	5,720	7	47	40	6.2	5,400	6.55	13	11	22	10	49	9	19	7.0
17	39.14	5,720	7	47	10	6.2	4,940	6.55	16	9	21	9	51	10	18	6.9
18	39.20	5,720	7	47	10	6.2	5,300	6.50	20	9	19	8	49	7	19	7.4
19	38.93	5,720	7	47	10	6.2	4,720	6.55	11	7	16	8	45	8	16	6.8
20	39.31	5,720	7	47	10	6.2	5,130	6.65	12	8	25	9	50	9	19	7.7
21	39.13															

* On this day patient vomited 2 L. of fluid containing 4 mEq. of Na, 12 mEq. of K, 28 mEq. of Cl, and 2 Gm. of nitrogen.

† Intake figures corrected for rejected diet.

‡ 40 mEq. HCl given in drinking water.

§ 30 mEq. HCl given in drinking water.

TABLE III—*Continued*

Day	Body wgt.	Stool				Plasma						
		Na	K	Cl	N	pH	HCO_3	Na	K	Cl	P	Creat.
	Kg.	*mEq./day*	*mEq./day*	*mEq./day*	*Gm./day*		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*
1	42.82	1	2	1	0.4	7.41	29.3	146	3.4	105	2.6	1.0
2	42.15	1	2	1	0.4	7.39	24.9					
3	41.54	1	2	1	0.4	7.40	23.2	143	3.8	111	3.2	
4	41.05	1	2	1	0.4	7.31	21.7					
5	41.03	1	2	1	0.4	7.32	21.2					
6	40.69	1	2	1	0.2	7.32	18.0	139	3.9	110	4.2	
7	40.83	1	2	1	0.2	7.32	17.7					
8	40.93	1	2	1	0.2	7.33	18.2	133	4.4	104	3.9	
9*	40.33	1	2	1	0.2	7.32	18.6					
10	40.00	1	2	1	0.2	7.31	15.9	134	5.2	104		
11	40.14	1	2	1	0.2			130	3.8	101	3.5	
12	40.35	1	2	1	0.2	7.32	19.3					
13	39.90	1	2	1	0.2	7.27	16.2	129	3.4	100	4.1	
14	40.02	1	2	1	0.2	7.35	16.0	129	3.7	100	3.4	
15‡	39.90	1	2	1	0.2	7.30	16.8	127	3.7	100		
16§	39.51	1	2	1	0.2	7.32	13.9	127	3.7	101		
17	39.14	1	2	1	0.2	7.26	11.2	128	3.7	104	3.5	
18	39.20	1	2	1	0.2	7.25	12.3	126	3.4	108	3.4	
19	38.93	1	2	1	0.2	7.28	13.0	126	3.5	108	3.4	
20	39.31	1	2	1	0.2	7.25	11.9	124	3.7	105	3.4	
21	39.13					7.30	12.2	124	3.5	105		0.9

8 to 25 mEq. per day, the average being 12 mEq. per day. In all patients except P.D., there was a slight tendency for ammonium excretion to increase as the urine pH fell.

Phosphorus. Phosphorus excretion in the entire group ranged from 10 to 24 mM per day. In the course of any single study, there was no significant change in phosphorus excretion either during the spontaneous development of acidosis or after administration of acid or sodium chloride.

Sodium excretion. Figure 6 shows the daily urinary sodium excretion for Patients P.D., D.H., H.G. and R.T.A., all of whom had a sodium intake of 7 to 12 mEq. per day. The level of the cross-hatched area at the bottom represents the average sodium intake of 9 mEq. per day. It is apparent that initially sodium excretion significantly exceeded intake but decreased rapidly towards intake values within the first week. However, in two subjects, P.D. and H.G., there was a continued excretion of sodium in excess of intake. The irregular line connecting Days 9 and 10 in P.D. signifies the actual passage of seven days during which time the balance study was interrupted because of vomiting. The patient was given approximately 100 mEq. of extra sodium each day during this interval, but was put back on an intake of 9 mEq. of sodium when the balance was resumed. It is clear, however, that in P.D., as well as in H.G., renal conservation of sodium was defective. In the other two subjects, sodium excretion slowly dropped to levels virtually equal to the intake. Stool sodium losses were negligible, but in view of the unmeasured skin losses of sodium, a continued urinary excretion of even 8 to 10 mEq. per day probably represented a net negative balance. Furthermore, during this period serum sodium in R.T.A. had fallen to 124 mEq. per L. (Table III).

R.M., whose sodium intake was 52 mEq. per day during the first 10 days of study, had a slight but persistent excess of sodium in his urine during the last few days of this period. When supplementary salt was given in the latter part of the study, the sodium balance became positive.

Chloride. Urinary chloride excretion exceeded intake during the first five to seven days after the patients were placed on a low-salt diet, but subsequently excretion and intake were virtually equal. R.M., whose chloride intake was 55 mEq. per day during most of his study, also excreted chloride in amounts approximately equal to his dietary intake.

Following administration of HCl or NH_4Cl (P.D., D.H. and R.T.A.) there was no increase in chloride excretion despite a rise in serum chloride concentration to between 108 and 111 mEq. per L.

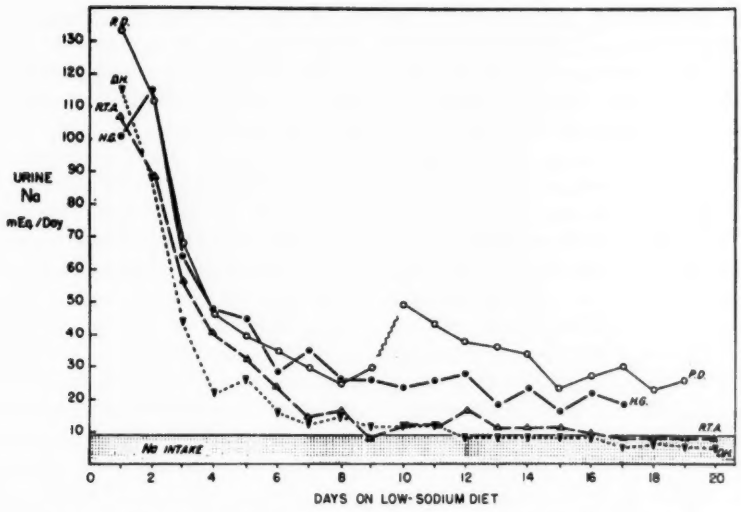

FIG. 6. DAILY EXCRETION OF SODIUM IN FOUR PATIENTS WITH RENAL DISEASE AND ACIDOSIS, THREE WITH UREMIA AND ONE (R.T.A.) WITH RENAL TUBULAR ACIDOSIS

The observations began the day after acidosis had been corrected and treatment with sodium bicarbonate had been stopped. The height of the cross-hatched area at the bottom indicates the average sodium intake of the four patients (range, 7 to 12 mEq. per day). The irregular line joining Days 9 and 10 for Patient P.D. indicates an interruption of seven days (see text).

Potassium. Patients P.D. (second study), D.H., H.G. and R.T.A. excreted potassium in amounts approximately equal to their intake, and there was little or no cumulative negative potassium balance during the course of study. R.M. and P.D. (first study) showed a urinary excretion of potassium consistently greater than intake, and cumulative negative potassium balances of 194 and 147 mEq., respectively. When potassium balance was corrected ($N \times 2.7$ mEq.) for the small negative nitrogen balance observed in each of these two subjects, the cumulative balance values were 180 and 116 mEq., respectively.

Stool electrolytes. Stool electrolyte content for the group as a whole was as follows: sodium, 1 to 3 mEq. per day; potassium, 2 to 12 mEq. per day; chloride, 1 to 4 mEq. per day.

DISCUSSION

The defect in bicarbonate conservation found in three of the four patients with uremic acidosis is noteworthy, in view of the scant attention accorded this problem in the past. It is recognized that patients with renal tubular acidosis and the Fanconi syndrome may show an inappropriate excretion of bicarbonate at subnormal plasma concentrations (11–13), and a similar defect has recently been described in the tubular nephropathy associated with Wilson's disease (15). However, this has been considered an abnormality confined essentially to the tubular nephropathies. Only one patient with uremia has been reported who seemed to waste bicarbonate (9), and Roberts, Randall, Vanamee and Poppell found no evidence of impaired reabsorption of bicarbonate in chronic renal insufficiency (10) when they acutely loaded such patients with alkali.

The difference between the present observations and those of most previous workers can probably be accounted for by several factors. In the first place, since patients with degenerative renal disease and bicarbonate-wasting may elaborate an acid urine virtually free of bicarbonate when the plasma bicarbonate is at or below the new

threshold level, the existence of the defect could readily be overlooked. Bicarbonate-wasting would not be evident unless such patients were receiving alkali. Secondly, it seems likely that many patients with uremia and acidosis do *not* have a bicarbonate-wasting defect. Of the group of four patients with uremic acidosis reported here in detail, all but R.M were found to have some significant degree of bicarbonate-wasting. However, of an additional group of eight acidotic, uremic patients studied without complete balances, only two were found to excrete significant quantities of bicarbonate after plasma levels had dropped below 24 mEq. per L. (16). Thus, of a total of 12 patients, bicarbonate-wasting was found in only five, and it is clear that the defect is often absent in uremic acidosis.

In the acute studies of Roberts and associates (10) mentioned above, difficulties inherent in the accurate measurement of very low filtration rates may have introduced methodologic uncertainties which would make the calculation of bicarbonate reabsorption subject to significant error. Finally, as will be pointed out below, it is possible that in acidotic patients with bicarbonate-wasting, loading with bicarbonate may of itself raise the apparent threshold and may, therefore, give higher values than those observed during acidosis.

The present study does not elucidate the mechanism leading to defective conservation of bicarbonate, but a possible explanation may be suggested. Renal disease may reduce carbonic anhydrase activity in tubular cells to the point at which the enzymatic reaction becomes the rate-limiting step in the process of reabsorption (17). The situation may be comparable to that which occurs when carbonic anhydrase is partially inhibited by chronic administration of acetazoleamide (18, 19). Under these conditions, plasma bicarbonate level falls until a new balance is attained between available enzyme activity and the filtered load of bicarbonate. However, it has been shown in acute studies (17) that the rate of reabsorption during partial inhibition of carbonic anhydrase is a direct curvilinear function of the plasma bicarbonate level, possibly reflecting a Michaelis-Menten relationship between enzyme activity and substrate concentration (17). Thus, in the experiments of Roberts and co-workers (10) loading with bicarbonate might well have accelerated the rate of reabsorption and masked a lowered renal threshold.

The bicarbonate-wasting defect apparently plays a dual role in the genesis of the acidosis in these uremic patients. The excretion of bicarbonate in the urine, in the first place, represents a direct loss of alkali from body fluids and so leads to acidosis. In P.D., in whom the defect was severe, a total of approximately 260 mEq. of alkali was excreted in the first seven days, prior to establishment of acid-base equilibrium. Distributed through a volume estimated as 40 per cent of total body weight, such an alkali deficit would be expected to lower plasma bicarbonate by approximately 10 mEq. per L. Since the observed drop in plasma level during this time was 13 mEq. per L., it is clear that in this patient loss of bicarbonate in the urine contributed the major share in the development of acidosis. Similar calculations for Patients D.H. and H.G., in whom cumulative losses of bicarbonate were approximately 75 mEq., indicate that excretion of alkali probably accounted for approximately half of the observed fall in plasma bicarbonate.

Bicarbonate-wasting, however, has another important influence on the genesis of uremic acidosis. Impairment of bicarbonate reabsorption would result in persistence of an alkaline fluid in the distal part of the nephron where ammonium and titratable acid normally appear. Thus, in addition to producing alkali loss, bicarbonate-wasting, by limiting the output of ammonium and titratable acid, contributes to acidosis by reducing the renal excretion of hydrogen. It is to be noted, in each of the first three figures, that acid excretion was suppressed during the early bicarbonate-wasting phase of each balance. In R.M. (Table II and Figure 4) there was essentially no bicarbonate loss and hence no direct contribution by this factor to the development of acidosis. However, as will be pointed out below, he, as well as all the other uremic patients, showed an abnormal lag in acidification of the urine which contributed to the delay in achieving acid-base equilibrium.

That patients with uremia and acidosis can ultimately acidify their urine had been noted by earlier workers (2, 3) and has recently been confirmed (20). In the present group minimal pH

values of 4.8 to 5.5 eventually were achieved. Since the major urinary buffer is phosphate (pK′ approximately 6.8), it follows that in urines of this acidity excretion of titratable acid must be nearly maximal. It would appear, however, that uremic patients require a more severe degree of acidosis to achieve this level of urine acidity than do normal subjects given ammonium chloride. In all the uremic patients, the lowest urine pH was achieved only after plasma bicarbonate levels had been depressed, spontaneously or with acid loads, to concentrations of 10 to 15 mEq. per L. (In the bicarbonate-wasting patients minimal urine pH did not develop until plasma levels were below those at which overt wasting had ceased.) By contrast, normal subjects loaded with NH_4Cl produce urine of comparable acidity when their plasma bicarbonate concentrations are in the range of 20 to 25 mEq. per L. (4, 21, 22).

A delay in acidification of the urine was apparently the major factor preventing Patient R.M. from initially excreting the maximal ammonia and titratable acid of which he was capable. The cumulative deficiency in the net excretion of acid (ammonia plus titratable acid) during the first 13 days of his balance (Figure 4), as compared to the average acid excretion on the last two days, amounted to 257 mEq. This is approximately two-thirds of the total amount calculated to be necessary to produce the observed drop in plasma bicarbonate during the first 13 days. Inefficiency in the acidifying process, with consequent delays in achievement of maximal excretion of titratable acid and ammonia, may well have been largely responsible for the development of acidosis in the other uremic patients who did not have any overt bicarbonate-wasting defect.

It should be emphasized, however, that all uremic patients appear to have an underlying defect in ammonium excretion, an abnormality which has long been recognized (2–7). Despite the progressive acidity of the blood and urine in these patients, excretion of ammonium rose only slightly. The present data emphasize the fact that this defect in ammonium excretion cannot be attributed to an unfavorable pH gradient for the accumulation of ammonium in the distal tubule. While the reduction in ammonium excretion may be the result of specific tubular dysfunction, it is equally possible that it is simply the consequence of the loss of tubular mass or of a critical reduction in tubular blood supply which limits the availability of substrate or the removal of by-products.

As a result of the impairment of ammonium excretion, this moiety constitutes only a third or less of the total acid excretion in the four patients with uremia (Figures 1 to 4). In normal subjects, by contrast, ammonium is usually one and one-half to two times the excretion of titratable acid, and in severe metabolic acidosis, ordinarily constitutes more than three-quarters of the total acid excretion.

With the development of acidosis and reduction of urine pH to relatively low levels, it can be assumed that the uremic patients are excreting acid at close to their maximal rates. It is clear that under these circumstances they are at the mercy of whatever additional exogenous or endogenous acid loads may be imposed. Thus, for example, when the plasma bicarbonate level was acutely reduced from 15 to 10 mEq. per L. in P.D. (Figure 1) acid excretion did not rise significantly and bicarbonate concentration remained fixed at this new low level for the next four days. Actually, as shown in Table I and Figure 1, there was a further reduction in urine pH (from 6.20 to 5.55) but, because the control pH was already well below the pK′ of phosphate, the increment in titratable acid was negligible. In Patient D.H. (Figure 2), it is not clear whether urine pH and plasma bicarbonate had reached their spontaneous minima prior to administration of the ammonium chloride load. However, in this case a reduction in urine pH from 6.5 to 5.2 was enough to permit a small, though appreciable, increase in excretion of titratable acid and ammonium. This produced a slow restitution of plasma bicarbonate level to its preloading concentration. The response, though ultimately effective, was clearly less than normal. Had still more acid been superimposed upon the rather modest load administered, it is quite likely that no further renal adjustments would have been possible, since the urine was now almost maximally acid.

Are these patients in acid-base equilibrium when their plasma bicarbonate concentrations have stabilized at lower than normal levels?

Since there is no method of computing the total hydrogen ion load, it is difficult to be certain whether renal acid excretion actually accounted for total hydrogen production. It is possible that slow, continuous titration of bone buffers as a result of a reduction in extracellular pH may have contributed to the stabilization of the plasma bicarbonate concentration. This appears to be the case in patients with renal tubular acidosis where there is much evidence to suggest the continuous gradual dissolution of bone salts (23), but whether this also occurs in uremic acidosis has not been clearly established. Analysis of bone in adults dying of uremia has so far failed to indicate significant changes in cation content (24), despite the fact that osteomalacia and demineralization of bone are occasionally quite apparent in chronic uremia (25). The role of bone buffers in the stabilization of extracellular pH in uremic acidosis therefore remains to be elucidated.

The observations on the patient with renal tubular acidosis (R.T.A., Figure 5 and Table III) have been reserved for separate consideration because the tubular defects in this condition appear to be characteristic and in part distinguishable from those found in patients with uremic acidosis. Previous workers have noted that in renal tubular acidosis there is a defect in acidification of the urine (8) as well as a tendency to waste significant amounts of bicarbonate at subnormal plasma concentrations (11–13). These two defects are well illustrated in Patient R.T.A., who excreted a total of 162 mEq. of bicarbonate during the first five days of the study, as her plasma level fell from 29 to 18 mEq. per L. Thereafter she continued to excrete an average of 15 mEq. per day and her urine pH never dropped below 6.5, even when the plasma bicarbonate was further depressed to 12 mEq. per L. by oral administration of HCl. In another adult with typical renal tubular acidosis and nephrocalcinosis similar observations have been made, but without benefit of balance studies (16). In this latter patient a total of 68 mEq. of bicarbonate was excreted over three days as the plasma level fell from 26 mEq. per L. to 16 mEq. per L. following withdrawal of alkali. Thereafter, with the plasma level remaining steady, this patient continued to excrete approximately 10 mEq. of bicarbonate per day in a urine of pH 6.4 to 6.7.

Although excretion of titratable acid was low, both patients were able to excrete normal amounts of ammonium relative to urine pH (13, 20). This pattern contrasts with that found in uremic acidosis where, under the proper stimulus, maximal excretion of titratable acid will occur, but ammonium excretion is persistently low. In both types of renal disease, however, bicarbonate-wasting may be a significant factor in the genesis of the acidosis. Thus, while the exact defects in these two disorders may vary, their general nature is the same, and consists essentially of a disturbance in acid (hydrogen) excretion or bicarbonate reabsorption, or both. Since these are entirely tubular functions, both types of acidosis should properly be considered as "tubular" in origin. A possible explanation for the difference in plasma anion pattern between uremic acidosis and renal tubular acidosis has been presented elsewhere (1).

Since reabsorption of bicarbonate is thought to involve sodium-hydrogen exchange, it is of interest to consider how the renal conservation of sodium in these patients might be related to their renal handling of bicarbonate. It is reasonable to assume that a part of the initial excretion of sodium during the first few days of sodium and bicarbonate withdrawal was directly linked with the bicarbonate-wasting (Figure 6). In Patients D.H. and R.T.A. sodium excretion dropped to very low values as the bicarbonate-wasting phase ended. In P.D. and H.G., however, significant wasting of sodium continued long after bicarbonate had virtually disappeared from the urine. It is therefore apparent that sodium-wasting does not require simultaneous loss of bicarbonate.

SUMMARY

In an effort to define the mechanisms responsible for the development of acidosis in patients with chronic renal failure, balance studies have been carried out in four patients with uremic acidosis and in one with renal tubular acidosis. Each study began with the restoration of the plasma bicarbonate concentration to normal by administration of sodium bicarbonate. Thereafter, no alkali was given and the patients were allowed to develop acidosis spontaneously. In

some cases challenging loads of acid were administered after the patients had reached subnormal but steady plasma bicarbonate concentrations.

During the phase of developing acidosis, three of the four patients with uremia were found to excrete significant quantities of bicarbonate in their urine even after plasma levels had fallen well below normal. Bicarbonate excretion virtually ceased only when plasma concentration had reached 15 to 20 mEq. per L. This loss of alkali in the urine accounted for most of the initial rapid reduction in plasma level in one patient, and approximately half of the drop in the other two. The bicarbonate-wasting defect was not present in the fourth uremic subject nor in the majority of other uremic patients studied less completely.

The other factor apparently responsible for the development of acidosis was a suppression of the excretion of ammonium and titratable acid, which appeared to be due in part to the relative alkalinity of the urine during the bicarbonate-wasting phase. There was, in addition, a delay in the acidification of the urine which occurred even when bicarbonate-wasting was absent or had ceased. However, each of the uremic patients, when severely acidotic, was ultimately able to lower urine pH below 5.5 and therefore to excrete nearly maximal amounts of titratable acid. Even in the most acid urines ammonium excretion increased only slightly. Most of the renal excretion of acid responsible for the maintenance of a low but steady plasma bicarbonate level was therefore accounted for by titratable acid. Two of these patients were unable to excrete any significant part of additional challenging loads of acid since titratable acid and ammonium were already nearly maximal.

The patient with renal tubular acidosis not only wasted bicarbonate in the urine, but also continued to excrete an alkaline urine even at low plasma bicarbonate levels. In this patient, ammonium excretion was not significantly reduced relative to urine pH and therefore accounted for most of the excretion of hydrogen in the steady state.

It is concluded that in patients with acidosis due to renal disease, defects in the tubular reabsorption of alkali or in tubular excretion of hydrogen, or both, are at fault. The relative importance of these disorders may vary, but in general terms, all renal acidosis is "tubular" acidosis.

REFERENCES

1. Schwartz, W. B., and Relman, A. S. Acidosis in renal disease. New Engl. J. Med. 1957, **256**, 1184.
2. Henderson, L. J. A critical study of the process of acid excretion. J. biol. Chem. 1911, **9**, 403.
3. Palmer, W. W., and Henderson, L. J. A study of the several factors of acid excretion in nephritis. Arch. intern. Med. 1915, **16**, 109.
4. Gamble, J. L., Blackfan, K. D., and Hamilton, B. A study of the diuretic action of acid producing salts. J. clin. Invest. 1925, **1**, 359.
5. Van Slyke, D. D., Linder, G. C., Hiller, A., Leiter, L., and McIntosh, J. F. The excretion of ammonia and titratable acid in nephritis. J. clin. Invest. 1926, **2**, 255.
6. Linder, G. C. The effect of mineral acid on acid-base regulation in health and in nephritis. Quart. J. Med. 1927, **20**, 285.
7. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry, vol. I, Interpretations, 1st ed. Baltimore, Williams & Wilkins Co., 1931.
8. Mudge, G. H. Clinical patterns of tubular dysfunction. Amer. J. Med. 1958, **24**, 785.
9. Peterson, V. P. Renal function and electrolyte metabolism in "salt-losing nephritis." Acta med. scand. 1956, **154**, 187.
10. Roberts, K. E., Randall, H. T., Vanamee, P., and Poppell, J. W. Renal mechanisms involved in bicarbonate absorption. Metabolism 1956, **5**, 404.
11. Stapleton, T. Idiopathic renal acidosis in an infant with excessive loss of bicarbonate in the urine. Lancet 1949, **1**, 683.
12. Latner, A. L., and Burnard, E. D. Idiopathic hyperchloraemic renal acidosis of infants. Quart. J. Med. 1950, **19**, 285.
13. Milne, M. D., Stanbury, S. W., and Thomson, A. E. Observations on the Fanconi syndrome and renal hyperchloraemic acidosis in the adult. Quart. J. Med. 1952, **n.s. 21**, 61.
14. Relman, A. S., and Schwartz, W. B. Effect of DOCA on electrolyte balance in normal man and its relation to sodium chloride intake. Yale J. Biol. Med. 1952, **24**, 540.
15. Bearn, A. G., Yü, T. F., and Gutman, A. B. Renal function in Wilson's disease. J. clin. Invest. 1957, **36**, 1107.
16. Relman, A. S., Hall, P. W., Hays, R. M., and Schwartz, W. B. Unpublished observations.
17. Schwartz, W. B., Falbriard, A., and Relman, A. S. An analysis of bicarbonate reabsorption during partial inhibition of carbonic anhydrase. J. clin. Invest. 1958, **37**, 744.
18. Leaf, A., Schwartz, W. B., and Relman, A. S. Oral administration of a potent carbonic anhydrase inhibitor ("Diamox"). I. Changes in electrolyte

and acid-base balance. New Engl. J. Med. 1954, **250**, 759.

19. Counihan, T. B., Evans, B. M., and Milne, M. D. Observations on the pharmacology of the carbonic anhydrase inhibitor "Diamox." Clin. Sci. 1954, **13**, 583.
20. Davies, H. E. F., and Wrong, O. Acidity of urine and excretion of ammonium in renal disease. Lancet 1957, **2**, 625.
21. Schwartz, W. B., Jenson, R. L., and Relman, A. S. The disposition of acid administered to sodium-depleted subjects: The renal response and the role of the whole body buffers. J. clin. Invest. 1954, **33**, 587.
22. Wood, F. J. Y. Ammonium chloride acidosis. Clin. Sci. 1955, **14**, 81.
23. Albright, F., and Reifenstein, E. C., Jr. The Parathyroid Glands and Metabolic Bone Disease. Baltimore, Williams & Wilkins Co., 1948.
24. Pellegrino, E. D., and Farber, S. J. Human bone electrolytes in various disease states. Clin. Res. 1958, **6**, 259.
25. Stanbury, S. W. Azotaemic renal osteodystrophy. Brit. med. Bull. 1957, **13**, 57.

THE NATURE AND MECHANISM OF THE HEMOSTATIC BREAKDOWN IN THE COURSE OF EXPERIMENTAL HEMORRHAGIC SHOCK *

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A severe bleeding tendency, the most striking features of which are fibrinogenopenia and thrombocytopenia, has been reported in some obstetrical accidents, certain types of surgery, multiple replacement transfusions and other clinical conditions (1). The pathogenesis of this syndrome remains a matter of dispute, as various authors have stressed the role of either intravascular clotting or of fibrinolysis. Most likely, both mechanisms are important, although of different impact from case to case. Especially in obstetrical and surgical practice, one encounters instances where severe hemorrhage occurs because of major trauma to vessels or because of the presence of a large bleeding surface. In some of these patients, bleeding becomes progressively more severe, and the fibrinogenopenic syndrome develops. The role of hemorrhage itself, at least in aggravating the bleeding tendency and, in some cases, in possibly initiating fibrinogenopenia, has apparently not been fully considered. It is the purpose of this paper to discuss the hemostatic changes occurring in the course of severe hemorrhage with special reference to the pathogenesis of fibrinogenopenic states.[1]

Important changes of the coagulation mechanism during severe hemorrhage have been noted by many authors. Hewson (2) made the classical observation that "the blood which issued last coagulated first in animals and men being venesected." Von den Velden (3), while confirming this observation, thought that the hypercoagulability in the course of hemorrhage might be due to the entrance in the circulation of interstitial fluid carrying thromboplastic material. A different conclusion was reached by McClintock and Magers (4). They noted shortening of the clotting time in severely bled dogs. This, however, did not occur in previously splenectomized animals. Thus, they concluded that the splenic vasoconstriction which accompanies hemorrhage might cause entrance of thromboplastic agents within the blood stream. The shortening of the clotting time during hemorrhage has been confirmed more recently in cats (5) and in dogs (6). Animals have been shown to develop hypoprothrombinemia during and after bleeding (7), and widespread fibrin clots have been washed out from the pulmonary vessels of dogs following death due to irreversible shock (8). Parallel studies in man, performed in individuals who had suffered from recent severe hemorrhage, have given inconclusive results. No changes in the clotting time (9) performed by standard techniques, but prolongation of the sensitized clotting time (10), have been reported. Hypoprothrombinemia seems a fairly constant finding (9).

MATERIALS AND METHODS

A total of 45 New Zealand white rabbits of the average weight of 2.5 Kg. were used. The following technique was employed to induce hemorrhagic shock. An area of skin covering the central artery of the ear was first anesthetized by infiltration with 1 per cent Novocaine® solution to prevent reflex vasoconstriction. Forty ml. of blood per kiloweight was then removed by gentle suction in cooled syringes through a 20 gauge needle inserted into the central artery of the ear, in an average time of 15 to 30 minutes. This amount represented approximately 70 to 80 per cent of the calculated blood volume. The loss of such percentage volume of blood has been shown to induce shock in the rabbit (11). In our study, pre-

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[1] Fairly extensive unpublished work in dogs, showing essentially the same results as presented here, was completed in this Laboratory during 1956–57 by Hector Marin, Leo Gagliardi and Mario Stefanini.

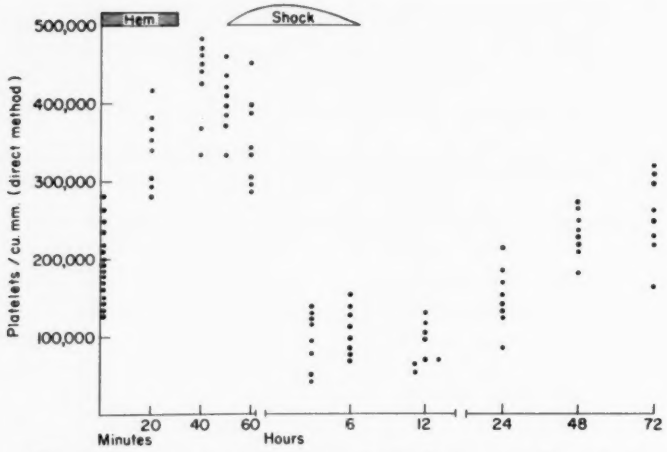

FIG. 1. BEHAVIOR OF THE PLATELET COUNT DURING AND FOLLOWING HEMORRHAGIC SHOCK IN RABBITS

Note the considerable variations from animal to animal, which, however, do not invalidate the uniform trend of the results. A similar consideration applies to all figures in this article.

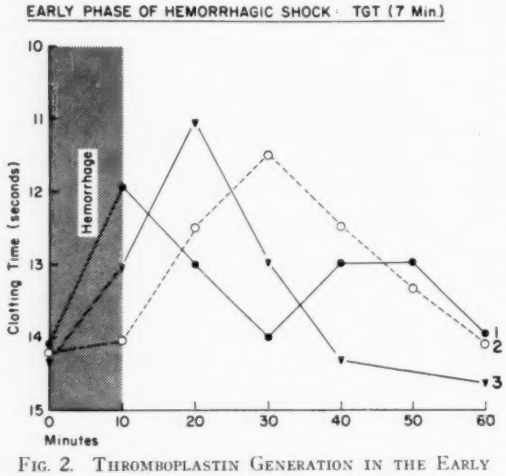

FIG. 2. THROMBOPLASTIN GENERATION IN THE EARLY PHASE OF HEMORRHAGIC SHOCK (SEE TEXT)

The values are those obtained after seven minutes of incubation of the reacting mixtures, and are from a single experiment. In each instance, platelets were obtained from human plasma for convenience, after preliminary experiments (R.A.T.) showed that their activity in the system could be substituted to that of rabbit platelets. Three experiments are described where plasma or serum from normal animals and animals in shock were used: 1) normal plasma and shock serum; 2) shock plasma and normal serum; 3) shock plasma and serum. Shock plasma and serum both seemed to accelerate the formation and increase the yield of thromboplastin, suggesting the presence of a clotting accelerator, the nature of which is under investigation.

sumptive indications of shock (prostration, extremely rapid heart rate) persisted for four to 10 hours. Blood was collected from the contralateral central artery of the ear before, during, and immediately and at various intervals of time after bleeding (every 10 minutes for the first hour and at 2, 4, 6, 24, 48 and 72 hours) until all values considered had returned to normal or near normal. Each time, 5 ml. of blood was collected in a siliconized syringe containing sodium oxalate at a final concentration of 0.01 M, and an additional 5 ml. in a plain glass syringe, for preparation of serum. The volumes of the test samples taken before, during and within the first 30 minutes of acute venesection were included in the calculation of the total volume of blood removed, as the continued bleeding probably contributed to the maintenance of the state of shock in the early phase of our experiments. Because of the technical difficulty involved in repeated venesections and also to prevent excessive removal of blood from a single animal, rabbits were divided into two groups. One group was studied in the early phase (up to one hour) and the other in the late phase (up to 72 hours). Studies included: *direct platelet count* (12) and observations of the morphologic appearance of platelets on a stained smear; *generation of thromboplastin* according to a standard technique (13) and using a modification with heterologous systems, where platelets were of human source and plasma and serum were supplied by an experimental or by a normal animal in various combinations; *activity of plasma prothrombin* (14); *concentration of prothrombin* (15) *and of stable factor* as proconvertin (factor VII) (15); *concentration of labile factor* (factor V) (16); and *determination of plasma fibrinogen level* (14).

The fibrinolytic activity of serum was studied with a number of techniques: *a*) A plate method, after addition

TABLE I

Thromboplastin generation test (TGT) during and following hemorrhagic shock in rabbits *

Incubation time of reagents for TGT	Time after initiation of bleeding									
	0	10 min.	30 min.	1 hr.	2 hr.	6 hr.	12 hr.	24 hr.	48 hr.	72 hr.
min.	*clotting times (seconds)*									
(A) Normal plasma and serum										
1	42.3 ± 6.2	41.4 ± 4.9	47.3 ± 5.1	46.3 ± 3.7	42.7 ± 2.9	44.3 ± 4.0	43.2 ± 2.8	49.4 ± 3.2	43.1 ± 3.4	41.9 ± 3.7
3	13.9 ± 1.6	14.2 ± 1.9	14.5 ± 2.0	14.1 ± 1.9	15.0 ± 0.4	14.2 ± 0.9	15.0 ± 0.5	14.0 ± 1.4	14.7 ± 0.7	15.3 ± 0.4
5	12.7 ± 1.7	12.5 ± 0.7	12.6 ± 1.1	13.1 ± 1.3	13.6 ± 0.8	13.9 ± 1.4	14.3 ± 2.0	13.9 ± 1.8	14.0 ± 1.6	13.2 ± 0.7
7	13.2 ± 2.4	13.0 ± 1.7	13.3 ± 2.1	13.7 ± 2.8	12.9 ± 2.0	13.2 ± 1.7	12.8 ± 1.7	13.4 ± 2.4	12.9 ± 2.1	13.1 ± 1.4
9	13.7 ± 2.1	13.5 ± 1.8	13.4 ± 0.9	14.1 ± 1.3	14.5 ± 2.3	14.2 ± 2.6	13.9 ± 0.8	14.3 ± 0.7	13.8 ± 1.2	14.1 ± 3.0
11	19.1 ± 4.2	20.4 ± 5.0	18.4 ± 3.2	20.7 ± 2.7	18.8 ± 2.3	19.4 ± 2.0	19.6 ± 2.4	20.3 ± 3.0	19.2 ± 2.0	21.6 ± 2.7
(B) Shock plasma and serum										
1	50.7 ± 5.1	47.3 ± 6.2	41.3 ± 7.0	48.2 ± 3.9	67.9 ± 4.6	69.3 ± 6.0	72.4 ± 8.2	64.9 ± 8.1	57.4 ± 7.7	47.4 ± 2.9
3	14.1 ± 0.7	13.0 ± 1.4	12.9 ± 0.9	16.7 ± 2.4	43.8 ± 3.7	40.1 ± 3.1	32.7 ± 4.0	24.7 ± 2.6	18.0 ± 1.4	13.9 ± 1.7
5	12.9 ± 1.7	12.6 ± 0.6	10.9 ± 0.7	13.2 ± 1.4	35.0 ± 5.0	27.4 ± 3.0	25.3 ± 4.7	21.4 ± 3.8	13.8 ± 0.5	13.0 ± 1.0
7	13.4 ± 0.5	12.3 ± 1.0	11.3 ± 0.6	14.3 ± 1.0	36.1 ± 4.1	29.0 ± 4.0	27.4 ± 3.2	19.6 ± 1.3	15.0 ± 1.7	13.2 ± 0.6
9	13.8 ± 1.2	13.0 ± 0.8	12.7 ± 0.9	15.1 ± 1.9	43.6 ± 6.3	32.4 ± 2.7	29.4 ± 4.2	24.3 ± 1.7	16.1 ± 1.2	13.4 ± 0.7
11	22.1 ± 2.9	19.2 ± 1.7	16.3 ± 0.9	24.6 ± 1.7	49.0 ± 3.8	43.7 ± 4.1	37.0 ± 4.0	28.4 ± 3.2	23.4 ± 1.9	20.7 ± 2.6
(C) Normal plasma, shock serum										
1	40.0 ± 3.7	34.2 ± 2.9	37.4 ± 3.8	41.0 ± 5.1	39.0 ± 2.7	47.0 ± 4.0	43.1 ± 4.1	42.7 ± 3.8	39.8 ± 3.7	42.3 ± 4.3
3	14.1 ± 0.6	11.7 ± 1.1	12.3 ± 1.2	14.2 ± 0.9	14.5 ± 1.3	13.7 ± 1.7	14.3 ± 1.0	13.8 ± 1.4	13.9 ± 0.9	14.9 ± 1.3
5	13.0 ± 0.4	10.9 ± 0.7	11.8 ± 0.9	13.2 ± 1.3	13.4 ± 1.4	12.9 ± 1.6	13.5 ± 1.9	12.8 ± 1.3	13.2 ± 2.0	13.0 ± 1.4
7	13.9 ± 1.1	11.4 ± 0.4	11.9 ± 1.3	13.8 ± 1.1	13.7 ± 2.0	13.3 ± 1.3	12.9 ± 1.7	13.4 ± 1.2	13.7 ± 1.8	13.0 ± 0.6
9	14.6 ± 1.0	12.8 ± 0.3	12.3 ± 0.7	13.7 ± 0.8	14.0 ± 1.2	14.7 ± 1.7	14.7 ± 2.0	14.3 ± 2.1	15.1 ± 0.9	14.7 ± 1.4
11	19.2 ± 2.3	16.4 ± 2.1	17.4 ± 2.7	22.1 ± 4.0	22.7 ± 4.3	19.0 ± 3.7	24.0 ± 5.7	23.6 ± 4.9	22.9 ± 3.7	23.0 ± 4.5
(D) Shock plasma, normal serum										
1	43.2 ± 3.2	39.0 ± 4.0	37.4 ± 2.7	39.2 ± 3.4	42.4 ± 4.1	38.9 ± 6.0	41.6 ± 2.8	42.4 ± 1.7	39.7 ± 3.7	40.9 ± 2.4
3	13.7 ± 1.7	13.0 ± 1.4	12.4 ± 0.7	14.0 ± 2.1	26.1 ± 3.9	28.4 ± 4.2	24.2 ± 3.0	19.9 ± 2.5	14.7 ± 2.9	15.0 ± 2.7
5	12.9 ± 1.4	12.4 ± 1.2	11.7 ± 1.3	13.4 ± 1.4	24.7 ± 5.1	21.7 ± 4.9	23.7 ± 3.2	17.5 ± 2.0	14.1 ± 0.9	13.2 ± 2.7
7	13.8 ± 0.7	13.0 ± 1.2	11.5 ± 0.8	13.0 ± 0.9	23.4 ± 2.4	22.4 ± 4.0	20.1 ± 3.2	16.3 ± 2.7	13.9 ± 0.9	13.5 ± 0.7
9	14.7 ± 1.7	13.8 ± 0.9	14.1 ± 1.4	15.4 ± 0.9	25.7 ± 5.0	24.8 ± 4.7	22.3 ± 3.9	18.0 ± 2.1	14.9 ± 1.6	14.8 ± 1.3
11	20.7 ± 2.8	18.4 ± 2.6	17.9 ± 2.0	22.0 ± 2.9	32.4 ± 4.1	29.3 ± 5.0	26.4 ± 4.1	23.0 ± 2.3	20.9 ± 3.2	21.4 ± 3.6

* Various combinations of plasma and serum were used. Human platelets, however, were used in all experiments. The figures given are averages and standard deviations for six experiments. One group of six rabbits provided data for the period from 0 to 2 hours; another for the period from 6 to 72 hours.

of streptokinase to serum (17). This technique essentially supplied information on the remaining profibrinolysin in the serum which could be activated by the addition of streptokinase. Thus, as used, it could probably be considered a test of utilization of profibrinolysin. *b*) Observation of the lytic activity of the rabbit serum when added directly to the fibrin plate (in a volume of 0.03 ml.). This technique and the following one gave information on the direct fibrinolytic activity of serum. *c*) A plasma clot method. Two-tenths ml. of serum was added to 0.3 of rabbit plasma diluted with an equal volume of isotonic saline solution. The mixture was promptly clotted by the addition of 0.1 ml. of thrombin solution containing 100 National Institutes of Health (NIH) units per ml. and the behavior of the clot followed at various intervals of time.

The *antifibrinolytic activity of serum* was studied by means of a modified plate technique.[2] Complement titer was determined by the method described in Boyd's monograph (18).

[2] *Outline of method for the study of the antifibrinolytic activity of rabbit serum.* The method is based on the ability of the antifibrinolysin of serum to prevent or oppose the digestion of a fibrin plate by activated fibrinolysin. *Reagents:* a) bovine fibrinogen prepared by the method of Milstone (19), 1.2 per cent solution in veronal saline buffer at pH 7.4; b) bovine thrombin (Parke, Davis), 100 NIH units in 1 ml. of isotonic saline; c) globulin fraction from human serum, 1 mg. per ml. of isotonic saline; d) streptokinase (Lederle) dissolved in veronal saline buffer (1 mg. in 1 ml.); e) rabbit serum, serially diluted with isotonic saline. *Technique.* Two per cent in volume of human euglobulin solution is added to a small volume of streptokinase solution. Two volumes of the mixture is then added to 98 volumes of fibrinogen solution. Ten ml. of the latter mixture is rapidly transferred to a Petri dish 9 cm. in diameter and immediately clotted by the addition of 1 ml. of thrombin solution. One drop (0.03 ml.) of each dilution of serum tested is then deposited gently on different areas of the plate, which is then incubated at 37° C. for 24 hours. At the end of incubation the plate is lysed except for the areas where serum has been deposited. *Evaluation.* The diameters of the surfaces where no lysis has occurred are measured and their areas calculated. The values obtained

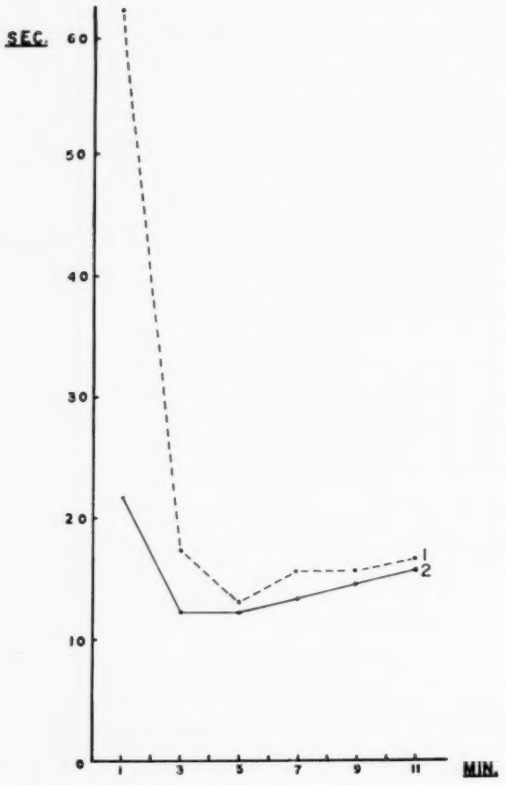

FIG. 3. THE CLOT ACCELERATING PROPERTY *IN VIVO* OF THE SERUM FROM A RABBIT IN THE EARLY PHASE OF HEMORRHAGIC SHOCK INJECTED INTO A NORMAL ANIMAL

Thromboplastin generation test, using the transfused animal's own plasma and serum, and human platelets (see legend to Figure 2). Figure shows thromboplastin generation 1) prior to injection of serum, and 2) 30 minutes after the injection of serum from the animal in hemorrhagic shock.

All animals were killed[3] 3, 24 or 96 hours after the establishment of hemorrhagic shock. Autopsy was performed immediately. After recording the macroscopic findings, thin slices of liver, lung, adrenal and kidney tissue were fixed in either Zenker's, or 10 per cent formalin solutions. Sections were then stained with hematoxylin-eosin, phloxine-methylene blue or phosphotungstic acid hemotoxylin stains.

RESULTS

I. Blood coagulation changes

It should be pointed out that the time relationship between hemorrhage and shock shown in the figures was somewhat arbitrary. In each instance, shock seemed to follow bleeding in short order of time, lasting between four and 10 hours after cessation of hemorrhage. Occasionally there was a more prompt recovery. In all cases, however, the changes observed in the coagulation mechanism showed a similar trend. Their behavior allowed the distinction of three phases: *a*) an early, *b*) a late and *c*) a recovery phase.

a) *Early phase.* This lasted approximately one hour and, thus, at least one-half hour be-

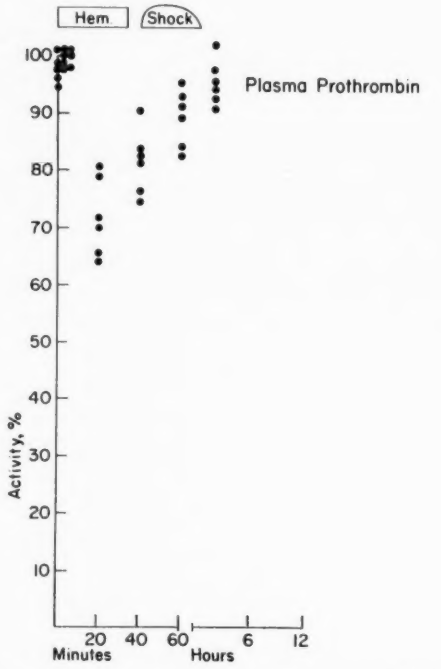

FIG. 4. PLASMA PROTHROMBIN PERCENTAGE ACTIVITY DURING HEMORRHAGIC SHOCK IN RABBITS (SEE TEXT)

Initial values, as for all figures, are prehemorrhagic values.

are compared with those obtained using various dilutions of a pool of serum from various normal animals. Undiluted pooled serum is conventionally assigned 100 per cent activity, and the various serial dilutions are assigned corresponding percentage values. Interpolation on logarithmic paper supplies direct reading values. Full details of the technique are being published elsewhere (20).

[3] One of the three following techniques was used: *1*) intra-arterial injection of approximately 30 ml. of air; *2*) intravenous injection of approximately 30 ml. of air; *3*) inhalation of $CH\,Cl_3$.

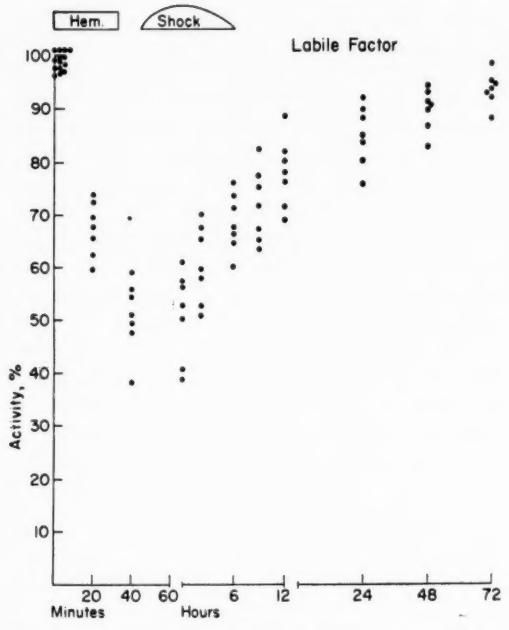

FIG. 5. PLASMA LABILE FACTOR ACTIVITY DURING HEMORRHAGIC SHOCK IN RABBITS (SEE TEXT)

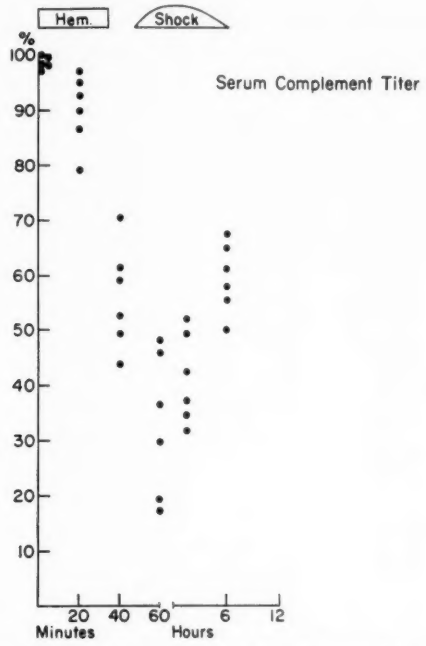

FIG. 6. SERUM COMPLEMENT TITER DURING HEMORRHAGIC SHOCK IN RABBITS
Results were entirely normal at 12 hours.

yond the termination of bleeding. Changes involved many of the clotting agents. Most significant perhaps was the development of a strong coagulation accelerating factor.

1) Platelets (Figure 1). Their number rose rapidly as bleeding began and doubled within 15 to 25 minutes. Platelets also became smaller and showed numerous dendrites.

2) Generation of thromboplastin. The speed of formation and total yield of thromboplastin were increased. Theoretically, this may be due to changes in platelets, plasma or serum. Platelets, being added in excess in all our tests, could be excluded in the interpretation of the results. As discussed in the legend to Figure 2 and shown

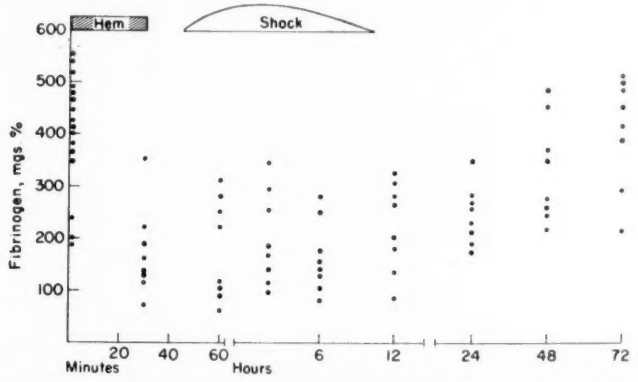

FIG. 7. PLASMA FIBRINOGEN LEVEL DURING AND FOLLOWING HEMORRHAGIC SHOCK IN RABBITS (SEE TEXT)

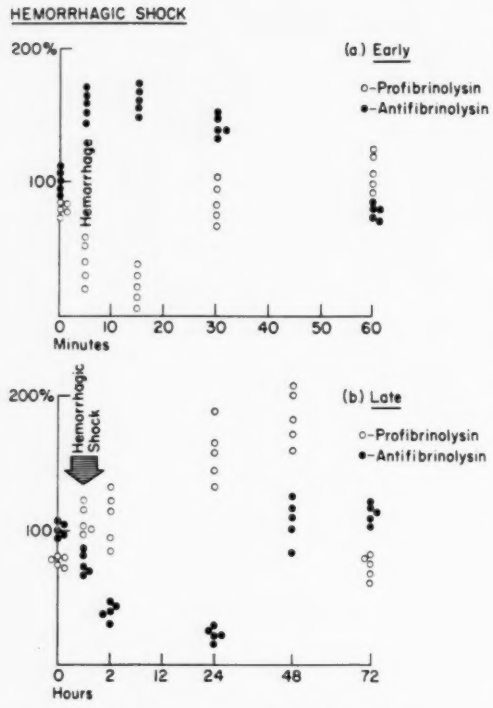

FIG. 8. THE FIBRINOLYTIC SYSTEM IN HEMORRHAGIC SHOCK: A) EARLY PHASE; B) LATE PHASE (SEE TEXT)

in Table I, however, the greater yield and accelerated formation of thromboplastin were due to changes in plasma and serum, both, but especially the serum, containing powerful activators. The activators were active *in vivo* as well as *in vitro*. Figure 3 illustrates an *in vivo* experiment to support this conclusion. Fifty ml. of blood was collected from a rabbit and the procedure repeated one hour later. Both samples were allowed to clot and the serum separated and incubated at 37°C. for four additional hours to make it free of thrombin activity. Each sample of serum was then injected into normal animals. Only moderate acceleration of the formation of thromboplastin followed the injection of the serum collected prior to bleeding; the acceleration was very marked after the injection of the serum collected during hemorrhagic shock.

3) Activity of plasma prothrombin (stable and labile factors). The prothrombin activity of plasma was only slightly affected (Figure 4). Studies on the activity of stable serum factor(s) involved in the conversion of prothrombin to thrombin (proconvertin activity) gave dubious results because of the presence of a strong clot-accelerating substance in the serum. The activity of plasma labile factor was significantly decreased (Figure 5). This is usually seen during activation of fibrinolysis or during active intravascular clotting. In the first instance, the agent is destroyed; in the second, it is converted to an accelerator which is found in serum. We have no data, at the present time, to conclude whether the clotting accelerator found in the serum of our experimental animals could be identified with "activated" labile factor. In any case, the presence of a clotting accelerator in plasma probably compensated for the drop in labile factor activity, thus explaining the overall minor change in the Quick plasma prothrombin time.

4) Serum complement.[4] There was a fairly short-lived fall in its titer (Figure 6).

5) Plasma fibrinogen level fell as soon as bleeding began. The low level of fibrinogen persisted for several hours after bleeding had been discontinued (Figure 7).

6) The fibrinolytic system (Figure 8a). There was a prompt drop in the profibrinolytic activity of serum and a corresponding rapid increment of the serum antifibrinolytic activity. There was at no time evidence of direct fibrinolytic activity of the serum on bovine fibrin plate or human plasma clots.

b) Late phase. This phase lasted between one and 48 hours after termination of hemorrhage, extending into the phase of recovery.

1) Platelets. After the initial rise in the early phase, their number fell rapidly, reaching a minimum value between 2 and 12 hours (Figure 1).

2) Generation of thromboplastin (Figure 9). This became slow and incomplete. An analytical study indicated that the defect in generation of thromboplastin was probably due to a complex combined depletion of the plasma and serum factors involved (Table I). It was noted that serum no longer accelerated the formation of thromboplastin when added to normal plasma and platelets.

[4] Determination performed by Miss Rose H. Mele.

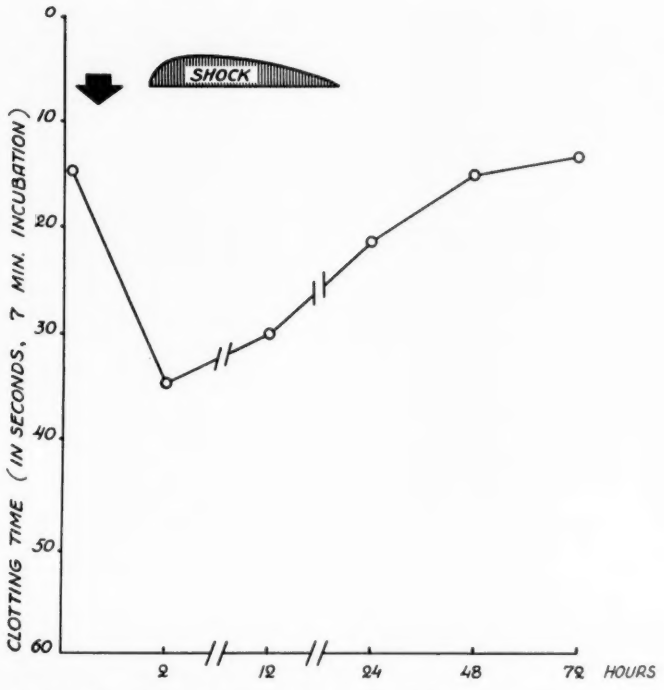

FIG. 9. THROMBOPLASTIN GENERATION TEST IN THE LATE PHASE OF HEMORRHAGIC SHOCK: A SINGLE EXPERIMENT

Results are those obtained at seven minutes of incubation of the reacting mixture, composed of the animal's serum and plasma and of human platelets.

3) Activity of plasma prothrombin and labile factor (Figures 4 and 5). The activity of plasma prothrombin, still slightly low at the beginning of this phase, rose rapidly to normal figures within a few hours. The activity of plasma labile factor remained below normal during this phase.

4) Plasma fibrinogen level remained very low for at least 12 hours after termination of bleeding, then began to rise (Figure 7).

5) The fibrinolytic system continued to show considerable changes (Figure 8b) for at least 48 additional hours. The findings were exactly the opposite of those observed in the early phase. The serum profibrinolytic activity was greatly increased, with a complementary fall in activity of serum antifibrinolysin. Again, no direct fibrinolytic activity could be demonstrated.

c) Recovery phase. This phase does not require a detailed description. All values returned to normal within 48 and 72 hours.

II. Anatomical and histological findings

Control animals showed no significant macroscopic findings, with the exception of occasional, small superficial petechiae and ecchymotic areas of the lungs. In contrast, animals which had undergone hemorrhagic shock showed evident areas of lung hemorrhage, decreasing in severity from the third to the ninety-sixth hour. The areas were often triangular in shape with the base toward the pleural surface of the lung and the apex on the hilus. At microscopic examination [5] of the lung in animals killed within three hours, the arterial vessels were generally collapsed with a small amount of blood in their lumina. There was

[5] We wish to thank Dr. Joseph J. Giammalvo, Department of Pathology, Tufts University School of Medicine, who kindly reviewed over two hundred sections and interpreted them. Dr. James H. Graham and Dr. Salvatore Allegra, Saint Elizabeth's Hospital, Boston, kindly helped in preliminary observations.

FIG. 10A. HISTOLOGIC FEATURES OF HEMORRHAGIC SHOCK IN RABBIT SACRIFICED THREE HOURS AFTER ESTABLISHMENT OF SHOCK

Pulmonary vessel, containing erythrocytes and "granules" of material with the staining properties of fibrin (phosphotungstic acid hematoxylin stain, × 400).

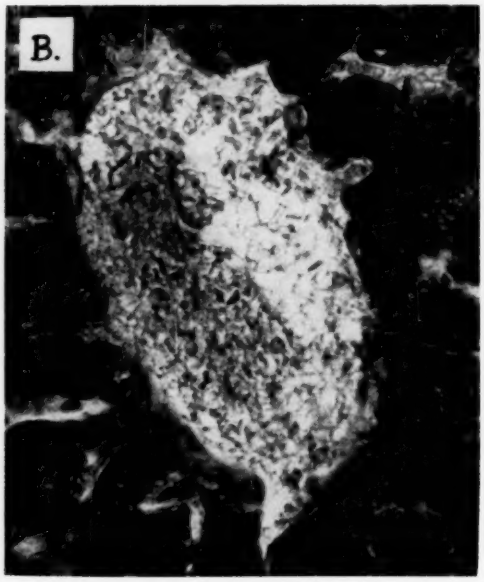

FIG. 10B. HISTOLOGIC FEATURES OF HEMORRHAGIC SHOCK IN RABBIT SACRIFICED THREE HOURS AFTER ESTABLISHMENT OF SHOCK

Hepatic vessel, containing erythrocytes, amorphous material and fine filaments of material with the staining properties of fibrin (phosphotungstic acid hematoxylin stain, × 800).

FIG. 10C. HISTOLOGIC FEATURES OF HEMORRHAGIC SHOCK IN RABBIT SACRIFICED THREE HOURS AFTER ESTABLISHMENT OF SHOCK

Pulmonary vessel containing thick fibers of fibrin (hematoxylin-eosin stain, × 120).

FIG. 10D. HISTOLOGIC FEATURES OF HEMORRHAGIC SHOCK IN RABBIT SACRIFICED THREE HOURS AFTER ESTABLISHMENT OF SHOCK

Magnification of C (× 400).

diffuse intra-alveolar hemorrhage, and moderately severe edema and focal atelectasis were generally present at the periphery. The tertiary bronchi were partially filled with blood within which clusters of fibrin were present. Some of the major veins were filled with blood mixed with fibrin clots. The histologic appearance of fibrin varied from granules (Figure 10a) to fine filaments (Figure 10b) to thick fibers (Figure 10c). The identification of these structures with fibrin was confirmed by their reaction with phosphotungstic acid hematoxylin and phloxinemethylene blue stains. Filaments and fibers were interwoven and surrounded granular masses of amorphous material resembling clumps of platelets. No organized thrombi were noted. A similar picture was observed within the venous vessels of liver and kidney. Findings were negligible after 24 hours and had regressed entirely after 96 hours.

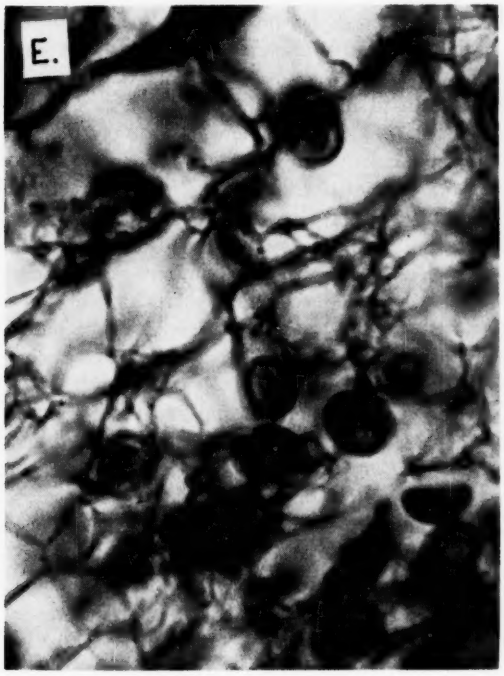

FIG. 10E. HISTOLOGIC FEATURES OF HEMORRHAGIC SHOCK IN RABBIT SACRIFICED THREE HOURS AFTER ESTABLISHMENT OF SHOCK

Hepatic vessel containing erythrocytes and fibrin fibers (phosphotungstic acid hematoxylin stain, × 1,500).

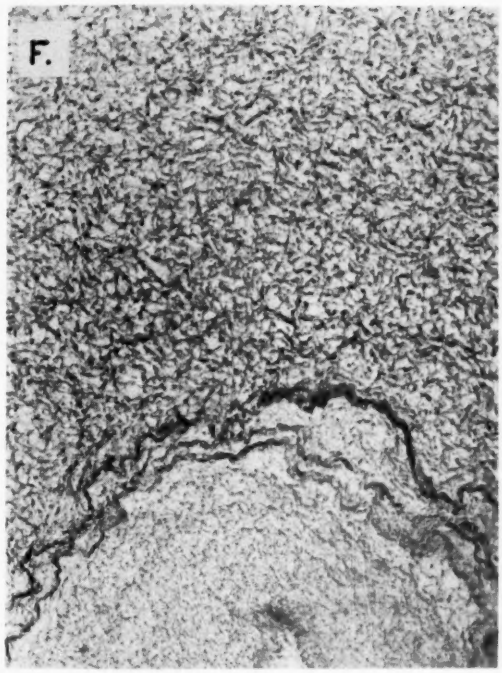

FIG. 10F. HISTOLOGIC FEATURES OF HUMAN PLASMA CONTAINING PLATELETS CLOTTED BY ADDITION OF BOVINE THROMBIN

Appearance of fibrin strands in the human plasma clot (hematoxylin-eosin, × 400). Note similarity to filaments in C.

III. Influence of splenectomy on the hemostatic breakdown of hemorrhagic shock

As mentioned, splenectomy prevents the acceleration of the clotting time in dogs during experimental hemorrhagic shock (4). Accordingly, it was decided to evaluate the effects of splenectomy on the changes in the coagulation mechanism of bled rabbits. Splenectomy was performed in four rabbits under pentothal anesthesia [6] by standard procedure. Three weeks after splenectomy, the same observations were carried out as for intact animals. Findings were much different. Platelets and fibrinogen level decreased only slightly during bleeding (possibly because of blood loss). Platelets and fibrinogen, in fact, rose markedly 24 and 48 hours later, respectively (Figure 11). There was decreased yield and

[6] Performed by Dr. Harman van Lessen (Bremerhaven, Germany) and Dr. Hector Marin.

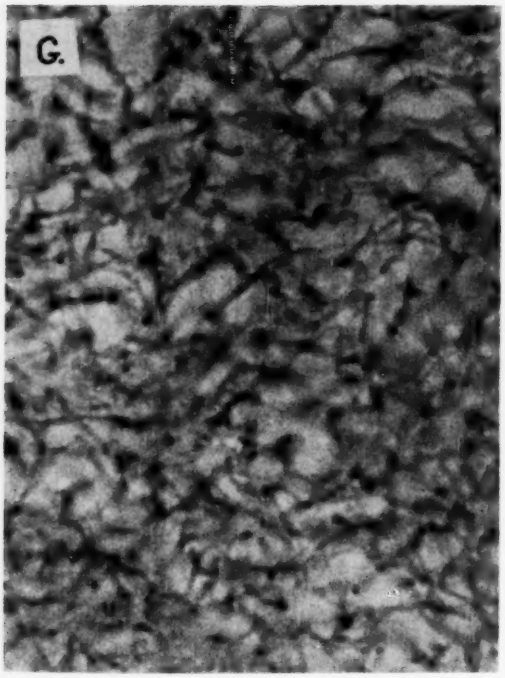

FIG. 10G. HISTOLOGIC FEATURES OF HUMAN PLASMA CONTAINING PLATELETS CLOTTED BY ADDITION OF BOVINE THROMBIN

Appearance of fibrin strands in the human plasma clot (× 1,200). Note similarity with findings in D.

speed of formation of thromboplastin for the first two to eight hours. The serum of the animal contained no clot accelerating agents as in the intact rabbits. Changes in activity of plasma prothrombin and labile factor were negligible. Fibrinolytic activity decreased slightly for many hours; the antifibrinolytic activity behaved in reciprocal manner to fibrinolysin (Figure 12). In any case, as discussed in the legends of Figures 11 and 12, changes were less significant than those observed in the intact animal. The autopsy performed three hours after bleeding showed no macroscopic hemorrhages in the lung or other organs. Microscopic sections of lung, liver and kidney showed numerous intravascular clumps of amorphous material which might have represented platelets. The findings were probably abnormal since they were not present in control animals. Yet no significant intravascular deposition of fibrin was seen, as in the intact animal.

DISCUSSION

The present work was originally planned to explain some of the abnormalities of the hemostatic mechanism previously observed during massive bleeding with multiple replacement transfusions in patients undergoing surgery (21, 22). A biphasic behavior was then recorded. Exaggerated and faster formation of thromboplastin, thrombocytosis, fibrinogenopenia and elevated antifibrinolytic activity were observed in an early phase. Severe breakdown of the hemostatic process followed, with marked thrombocytopenia, fibrinogenopenia and exalted fibrinolytic activity. It was felt that the findings might have been due to the effect of hemorrhage (21). The conclusion is supported by the results of the studies reported here, as the present findings correlate well with the previous clinical observations

An attempt was made in the course of this paper to divide the sequence of events into various phases (Figure 13). This was necessary since there were significant differences in findings at various intervals of time. This consideration might explain the discrepancies observed in the study of patients who develop hemorrhagic shock. In each phase, three significant factors were to be considered: *1*) the blood loss; *2*) the occurrence of intravascular clotting; and *3*) the rapid rate of production of clotting factors by the body and the transfer of proteins from tissue pools to the circulating plasma.

The blood loss seemed important in an early phase, since it induced rapid subtraction of clotting factors from the blood stream. Yet, a number of findings indicated that blood loss alone might not be invoked as an overall explanation. Fibrinogen and other clotting factors remained low for hours, when synthesis and shifting from tissue pools to plasma should have induced an opposite trend. More important, the accelerated rate of formation and increased yield of plasma thromboplastin, and the appearance of a clot accelerating factor during the early phase of hemorrhagic shock (the nature of which is being investigated) seemed to indicate "increased coagulability" of the blood. This hypothesis was further substantiated by the histologic findings of fibrin deposition within the blood stream. Thus, intravascular clotting was at least one (if not the most

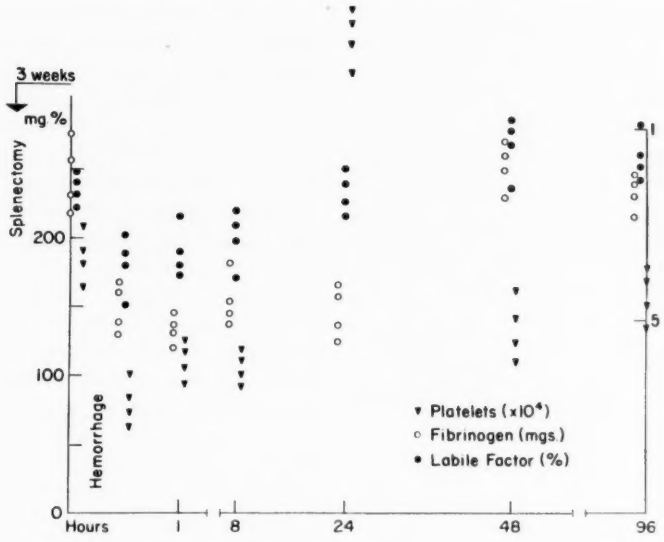

FIG. 11. THE COAGULATION FACTORS IN A SPLENECTOMIZED RABBIT DURING HEMORRHAGIC SHOCK

Using Figures 1 and 7 as comparison, note: a) the initial high platelet count, to be expected three weeks after splenectomy; b) the lack of early elevation of platelets and their remarkable rise during the late phase of hemorrhagic shock; and c) the fairly minor fall of fibrinogen level as compared to the extreme fibrinogenopenia of the intact animal.

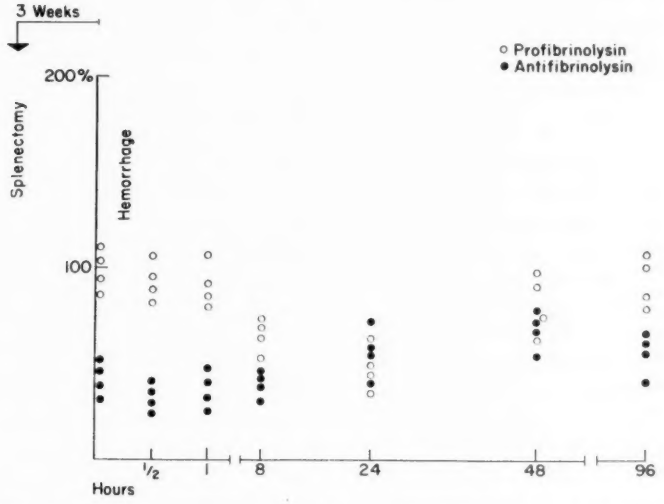

FIG. 12. THE FIBRINOLYTIC SYSTEM DURING HEMORRHAGIC SHOCK IN FOUR SPLENECTOMIZED RABBITS

Using Figure 8 as comparison, note: a) lack of significant changes in the early phase, and b) the minor, possibly insignificant, changes in the late phase.

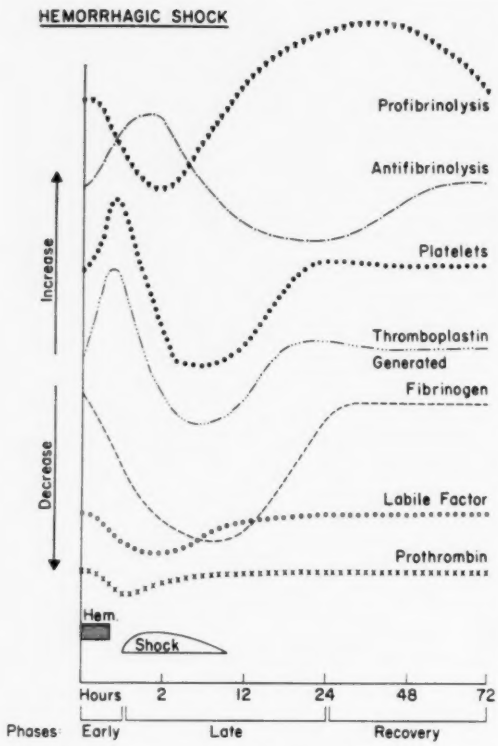

FIG. 13. HEMORRHAGIC SHOCK: A SUMMARY

This schematic figure shows the variations of many of the clotting factors in relation to time (see text). Curves are based on the mean values in 12 animals selected at random, six of which were studied in the early, and six in the late and recovery phases.

important) reason for further depletion of clotting factors observed in the late phase of hemorrhagic shock. The clotting agents might have been used in the formation of multiple intravascular clots which were observed in our study. Intravascular coagulation might have also played a role in the establishment of "irreversible shock." Why lung, liver (and kidney) were the organs where most of the deposition of fibrin was histologically evident cannot be explained with certainty. It could be related to the function of these organs as filters for the general and mesenteric circulation.

As it is generally assumed that the process of blood coagulation is a delicate balance between forces of clot formation and forces of clot lysis, it was not surprising that important changes in the fibrinolytic system occurred in hemorrhagic shock, together with the changes in activity, number and concentration of various clotting factors. These changes were difficult to interpret. There was a late rise in serum profibrinolytic activity which was, on the contrary, quite low in the early phase of shock. This suggested early consumption of profibrinolysin *in vivo* and, thus, activation of fibrinolysin which, then, may have followed in the wake of intravascular clotting. Antifibrinolysin had a reciprocal behavior, possibly neutralizing the fibrinolytic activity and thus explaining the lack of direct fibrinolytic activity of the sera *in vitro*. It is difficult to reject the temptation of interpreting these changes as expression of a defense mechanism against extensive thrombosis.

Finally, of great interest for the interpretation of the hemostatic changes associated with hemorrhagic shock was the failure of these changes to develop in animals whose spleen had been recently removed. There was no evidence of formation of clot accelerating factors nor of important changes in the fibrinolytic system, such as were observed in the intact animal. From a histologic viewpoint, there was no intravascular deposition of fibrin. Possibly, findings could be explained simply by the loss of formed blood elements and of clotting factors through bleeding. On the basis of these results, it appeared difficult to exonerate a mechanism of splenic contraction in the pathogenesis of the intravascular clotting of hemorrhagic shock. As already postulated by McClintock and Magers, the splenic contraction which followed bleeding might have caused emmission into the blood stream of a clot-promoting factor. This was likely to be a tissular splenic thromboplastin or a thromboplastic material from platelets lysed in the spleen.

SUMMARY

1. Rabbits bled until development of shock showed significant alterations of the hemostatic process. Various phases were recognized. A state of "hypercoagulability" of the blood developed at first, as indicated by thrombocytosis, accelerated generation of thromboplastin, and rapidly instituted fibrinogenopenia. Plasma loss was, at least in part, responsible for the latter (early phase). As the hemorrhagic shock deep-

ened, even after bleeding was stopped, there was a complete hemostatic breakdown. Platelets, fibrinogen and other coagulation factors were depleted. Fibrinolysis also developed, possibly as a mechanism of defense against intravascular deposition of fibrin (late phase). The various clotting factors later returned to normal values in variable periods of time if the hemorrhage was arrested and the state of shock regressed (recovery phase).

2. Histologic studies showed significant intravascular deposition of fibrin, possibly of platelets, in the vessels of lung, kidney and liver. This suggested widespread intravascular clotting.

3. Previous splenectomy mitigated the severity and duration of the hemostatic changes.

4. There was a significant correlation between these experimental results and some findings of the severe hemostatic breakdown in the course of hemorrhagic shock in man.

ACKNOWLEDGMENT

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REFERENCES

1. Stefanini, M. Fibrinolysis and "fibrinolytic purpura." Blood 1952, **7**, 1044.
2. Hewson, W. *in* The Works of William Hewson, F.R.S., G. Gulliver, Ed. London, Sydenham Soc., 1846, p. 46.
3. von den Velden, R. Blutverlust und Blutgerinnung. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1909, **61**, 37.
4. McClintock, J. T., and Magers, E. Coagulation time following hemorrhage after splenectomy. Proc. Soc. exp. Biol. Med. (N. Y.) 1926, **24**, 203.
5. Gray, H., and Lunt, L. K. Factors affecting the coagulation time of blood: V. Effects of hemorrhage before and after exclusion of abdominal circulation, adrenals, or intestines. Amer. J. Physiol. 1914, **34**, 332.
6. Shafiroff, B. G. P., Doubilet, H., Siffert, R., and Co Tui. Effect of hemorrhage on normal and hypocoagulable blood and lymphs. Amer. J. Physiol. 1943, **138**, 753.
7. Smith, J. J., Hartwick, J. P., and Regan, M. J. Coagulation mechanism in hemorrhagic shock (abstract). Fed. Proc. 1957 **16**, 120.
8. Crowell, J. W., and Read, W. L. *In vivo* coagulation —A probable cause of irreversible shock. Amer. J. Physiol. 1955, **183**, 565.
9. Warren, R., Lauridsen, J., and Belko, J. S. Alterations in numbers of circulating platelets following surgical operation and administration of adrenocorticotropic hormone. Circulation 1953 **7**, 481.
10. de Takats, G. Sensitized clotting time; simple test for bedside study of clotting mechanism. J. Amer. med. Ass. 1951, **146**, 1370.
11. Wiggers, C. J. Physiology of Shock. New York, Commonwealth Fund Publ., 1950.
12. Stefanini, M., and Dameshek, W. The Hemorrhagic Disorders. New York, Grune & Stratton Inc., 1955, p. 286.
13. Biggs, R., and Douglas, A. S. The thromboplastin generation test. J. clin. Path. 1953, **6**, 23.
14. Quick, A. J. The Hemorrhagic Diseases and the Physiology of Hemostasis. Springfield, Ill., Charles C Thomas, 1942, p. 312.
15. Owren, P. A. The diagnostic and prognostic significance of plasma prothrombin in factor V levels in parenchymatous hepatitis and obstructive hepatitis. Scand. J. clin. Lab. Invest. 1949, **1**, 131.
16. Wolf, P. A modification for routine laboratory use of Stefanini's method of estimating factor V activity in human oxalated plasma. J. clin. Path. 1953, **6**, 34.
17. Astrup, T., and Müllertz, S. The fibrin plate method for estimating fibrinolytic activity.. Arch. Biochem. 1952, **40**, 346.
18. Boyd, W. C. Fundamentals of Immunology, 3rd ed. New York, Interscience Publishers, Inc., 1956.
19. Milstone, J. H. Three-stage analysis of blood coagulation. J. gen. Physiol. 1948, **31**, 301.
20. Turpini, R., and Stefanini, M. In preparation.
21. Stefanini, M., Mednicoff, I. B., Salomon, L., and Campbell, E. W. "Thrombocytopenia of replacement transfusion": A cause of surgical bleeding (abstract). Clin. Res. Proc. 1954, **2**, 61.
22. Stefanini, M. Hemorrhagic tendency following massive transfusions at surgery. Proc. VIth Congress, Int. Soc. Blood Transfusion (Boston, Sept. 3, 1956). Basel, S. Karger, 1955, p. 78.

STUDIES OF IODOALBUMIN METABOLISM. I. A MATHEMATICAL APPROACH TO THE KINETICS*

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During the clinical study and treatment of a group of patients with thyroid carcinoma, we have studied the effects of thyroid hormone on several metabolic systems under conditions in which the thyroid status of the patients could be controlled at will. Early in the tracer studies of iodoalbumin metabolism in these patients it was apparent that it was necessary to employ a mathematical approach somewhat different from published methods of compartment analysis. Accordingly a suitable mathematical approach was developed by one of the present authors (1). In this report, the practical difficulties encountered in application of the approach to experimental data and the theoretical implications of the approach are discussed.

The kinetic analysis of the data of a single tracer study comprises the body of the report. Additional data from other tracer studies are included only insofar as they contribute to the development of the model chosen for analysis.

METHODS

General. The methods of dialysis of the commercial I^{131} labeled human serum albumin employed and details of the injection procedure, collection of blood and urine samples, quantitation of radioactivity in urine and plasma, determination of serum albumin and quantitation of total radioactivity removed in blood sampling have been given elsewhere (2). Stool collections were made only in the case of M. H. in Experiment III. The individual specimens were suspended in concentrated sodium hydroxide and distilled water to known volume, allowed to digest partly, and then agitated until a uniform pipettable mixture was obtained. The counting procedure was the same as that for urine and plasma (2). None of the patients studied showed albuminuria No chemical fractionation of label was done routinely on urine or plasma samples. In an ancillary study, Patient G. S., hospitalized because of breast carcinoma with bone metastases, but clinically euthyroid and without evidence of cardiac, renal or hepatic disease, was given 2 mc. of iodoalbumin intravenously and two 24 hour urine collections were made. Chemical fractionation with added carrier of the first urine showed 0.004 of the label precipitable with 5 per cent trichloracetic acid, 0.854 precipitable with 10 per cent silver nitrate, and 0.144 not precipitable with either agent. The respective figures for the second 24 hour urine were 0.004, 0.886 and 0.108. Paper chromatography showed the major constituents to be iodide (about 90 per cent) and diiodotyrosine (about 10 per cent). In the case of M. H., Experiment III, serum samples taken on Days 4 and 8 were subjected to paper electrophoresis. Virtually all of the label could be accounted for in the albumin fraction.

Selection of patients and description of experiments. The two experiments reported here (I and III) were carried out on five patients, all under observation for treatment of histologically proven thyroid carcinoma. All had previously had either surgical removal or I^{131} radiation destruction of their thyroids or both as part of their therapeutic regimen. Those patients developing severe hypothyroidism or myxedema were chosen for the iodoalbumin tracer studies.

Additional details of the clinical status of the patients and the experimental protocol for Experiment I are given in Reference (2). Features relevant to the present report follow.

Experiment I. This experiment was performed to study the metabolism of iodoalbumin in the euthyroid and myxedematous steady states in the same individual. Four patients were studied, each in both states on different occasions. Blood and urine samples were collected for 14 days following injection of the I^{131}-albumin. At the time of the first tracer study all had gross clinical evidence of myxedema. Otherwise they were fully ambulatory and in good general physical condition throughout both studies. In six studies the thyroid uptake of I^{131} was blocked either by an antithyroid drug or following cholecystography. In the two studies carried out without blocking agents the patients demonstrated their inability to retain I^{131} presented as iodide ion by excreting in four days 96 and 100 per cent, respectively, of the label administered as NaI^{131} in tracer studies carried out subsequent to the iodoalbumin studies but under the same metabolic conditions.

Experiment III. This experiment was performed for four reasons: *1)* to quantitate fecal excretion of label,

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2) to follow urinary excretion and plasma levels for a prolonged period during a steady metabolic state, *3*) to study in a recipient subject the metabolic behavior of iodoalbumin previously subjected to an eight day tenure in a donor subject, and *4*) to study carefully the early renal excretion of label in the recipient. It was thought that the donor would act as a biological "filter" and remove from the labeled material the more rapidly degraded constituents, if any were present, rendering a more homogeneous product for the recipient tracer study.

Two patients were studied. The donor, M. H., was an 81 year old female with known thyroid cancer and cranial metastases for nine years. She had received multiple I^{131} therapies in the previous four years with almost complete destruction of the cranial lesion but with persistence of a large cervical tumor. In addition she had hypertension and arteriosclerotic heart disease with angina pectoris but no evidence of congestive failure or hepatic or renal disease. Two months prior to the experiment her cholesterol was 364 mg. per cent and she was started on Tapazole®, 40 mg. daily. The latter was continued throughout the 63 days of the study during which time she exhibited evidence of myxedema. During the study her mean cholesterol was 328 mg. per cent; her mean basal metabolic rate (BMR), −37 per cent. Her weight varied from 65.1 Kg. at the beginning to 64.5 Kg. at the end of the study. A NaI^{131} tracer given after completion of the study showed a urinary excretion of 0.966 of the dose in 10 days.

The pertinent clinical data on R. B., the recipient, appear elsewhere (*2*). For one month prior to and during the study she received Tapazole®, 40 mg., and triiodothyronine, 150 μg., daily. She was also given saturated solution of potassium iodide, 15 minims daily, during the experiment. Her weight varied between 49.1 and 50.6 Kg. Her electrocardiogram was normal, her mean cholesterol 149 mg. per cent. Clinically she appeared mildly hyperthyroid.

On Day 1 of the study, M. H., the donor, was given intravenously 4.82 mc. of I^{131} iodoalbumin. Plasma samples were taken as previously described. All urinary voidings were collected separately for the first six days, after which 24 hour collections were made. On Day 8, 450 ml. of blood representing 281 ml. of plasma was taken by venepuncture under sterile precautions with simultaneous replacement transfusion. (The latter was considered mandatory in view of the donor's cardiovascular status.) The plasma was separated by centrifugation and three hours after venesection 240 ml. of the nondialyzed donor plasma of activity 37 μc. was given intravenously to R. B. The injection required 28 minutes. The first plasma sample was taken six minutes following completion of the injection.

All urinary voidings were collected separately for the first six days and then 24 hour collections were made. In the case of R. B. the study was continued for 20 days following injection. An intravenous NaI^{131} tracer performed after completion of the iodoalbumin study showed 0.966 urinary excretion of I^{131} in four days. Similarly an intravenous tracer of I^{131}-diiodotyrosine under the same regimen that existed during the iodoalbumin study showed 0.994 urinary excretion of I^{131} in five days.

In the case of R. B. the per cent probable error of counting of the least active plasma samples did not exceed 0.9 per cent; that of the least active urine samples, 3.4 per cent. For M. H. the respective figures were 0.8 and 1.2 per cent. For the stool samples of M. H. the per cent probable error did not exceed 6.4 per cent except in 14 samples toward the end of the study where the probable error was as high as 15 per cent.

Units and graphical representation of data. Plasma specific activity was expressed as fraction of the injected dose of radioactivity per gram of albumin. In Experiments I and III the variation in serially determined plasma albumin concentrations was within the error of the method.

Plasma specific activity was plotted against time (in hours) on semilogarithmic coordinates. In all cases these curves could be reduced to a minimum of three exponential components by standard methods of graphical analysis. The slowest component was obtained first by a least squares fit of the terminal linear portion of the plot. The remainder of the curve was smoothed by eye, and the two additional components were obtained by serial subtraction in the usual way (*3*). The standard errors of the slope and of the ordinate intercept of the slowest component were computed according to the method described by Snedecor (*4*). The exponential constant, α, for the slow component was available from the least squares calculations. The $T_{\frac{1}{2}}$ of each of the faster components was obtained directly from the graph and the exponential constant computed from the relationship: $\alpha T_{\frac{1}{2}} = 0.693$. The ordinate intercepts of the three components gave the coefficients, X_{ji}, of the exponential terms.

Urinary excretion data were plotted as cumulative fraction retained (1-cumulative excretion) on semilogarithmic coordinates. The cumulative fraction retained was corrected for fraction removed in blood sampling in Experiment III. In the case of M. H. the total fraction of the injected dose removed in blood sampling over the 63 days of the experiment was 0.0347, including 0.0177 removed to obtain labeled plasma for injection into R. B. In the case of R. B., Experiment III, 0.025 of the injected dose was removed in blood sampling over a 20 day period (larger blood samples were required because of the relatively small amount of radioactivity injected). In all other instances the fraction removed in sampling was less than 0.01 and was disregarded. In the case of M. H., Experiment III, the cumulative fecal excretion in 63 days was 0.0156. This was taken into account in computing the cumulative fraction retained in this instance, but fecal excretion was not measured and was disregarded in all of the other studies, which were of shorter duration.

The terminal, apparently linear, portions of the retention curves (beginning about the fourth or fifth day) were fitted by the method of least squares and the standard errors of the slopes and ordinate intercepts computed by the same approach that was used for the plasma curves.

RESULTS AND ANALYSIS OF DATA

Definitions

Carrier: The unlabeled form of the substance being studied.

Compartment: An aggregate of molecules which have the same transition probability from the state they are in to all other available states.

Iodoalbumin degradation: Alteration of the iodoalbumin molecule such that iodine in an excretable form is liberated.

Three-compartment series model: A three-compartment system in which the originally labeled compartment interacts with only one of the two remaining compartments.

Three-compartment parallel model: A three-compartment system in which the originally labeled compartment interacts with both the remaining compartments which in turn do not interact with each other.

A_{ji}: The coefficient of the i^{th} exponential term in the equation describing the fraction of injected tracer in the j^{th} compartment as a function of time. Graphically, an ordinate intercept.

α_i: Exponential constant (fraction per hour) in the i^{th} exponential term.

S_j: The amount of carrier albumin (grams) in the j^{th} compartment.

S_T: Sum of carrier (grams) in all compartments (total exchangeable albumin).

$q_j(t)$: Quantity of label (fraction of injected dose) in compartment j at time t.

$x_j(t)$: Specific activity (fraction of injected dose of label per gram of carrier) of compartment j at time t.

X_{ji}: Coefficient of the i^{th} exponential term in the equation describing the specific activity of the j^{th} compartment as a function of time. Graphically an ordinate intercept.

λ_{ij}: Fraction of S_j or $q_j(t)$ transferred to compartment i from compartment j per hour; a fractional turnover rate.[1]

λ_{oj}: Fraction of S_j or $q_j(t)$ leaving the exchanging system per hour by all avenues of unidirectional loss.[1,2]

λ_{jj}: Sum of all fractional rates of loss of S_j or $q_j(t)$ from compartment j per hour; the total fractional turnover rate of compartment j.

λ_{oT}: Fraction of S_T leaving the exchanging system per hour by all avenues of unidirectional loss.[1,2]

ρ_{ij}: Rate of transfer of carrier (grams per hour) to compartment i from compartment j.[1]

ρ_s: Steady state rate of synthesis of carrier (grams per hour).

K_{ie}: Fraction of iodide in the iodide space excreted by the kidneys per hour.

K_{de}: Fraction of diiodotyrosine in the diiodotyrosine space excreted by the kidneys per hour.

Development of a schematic model for kinetic analysis of data

Evidence has been presented by many investigators for the existence of an extravascular albumin compartment approximating in size the plasma albumin (5–7). The model proposed in this paper consists of plasma (Compartment 1), two extravascular exchanging compartments (Compartments 2 and 3), a compartment containing nonexcreted degradation products (Compartment 4), collected urine (Compartment 5) and an extravascular nonexchanging compartment (Compartment 6). Several arguments to justify the choice of this model follow.

The data of R. B., Experiment III, are presented graphically in Figure 1. Graphic analysis of the plasma curves indicated that in all the tracer studies plasma specific activity could be expressed as a function of time by an equation involving a minimum of three exponential terms:

$$1) \quad x_1(t) = X_{11}e^{-\alpha_1 t} + X_{12}e^{-\alpha_2 t} + X_{13}e^{-\alpha_3 t}.$$

The fact that three exponential components were necessary to fit the plasma curves requires that the system be composed of at least three compartments (1). The recovery of label in urine and feces indicates a system of the open type.

It can be shown (1) for a three-compartment steady state system, open or closed, that the specific activity or quantity of labeled material in each of the compartments may be expressed as a function of time by an equation involving three exponential terms, the exponential constants of which (α_i) are identical for all the compartments:

$$2a) \quad q_1(t) = A_{11}e^{-\alpha_1 t} + A_{12}e^{-\alpha_2 t} + A_{13}e^{-\alpha_3 t},$$

where

$$\sum_i A_{1i} = 1$$

[1] We wish to emphasize that the subscripts of λ_{ij} and ρ_{ij} are read "to i from j." This is a reversal of the directional sense of the subscript notation commonly appearing in the biological literature and was employed here because of greater convenience in matrix operations and to preserve notational consistency with Reference (1).

[2] Unidirectional loss, as used here, includes such processes as iodoalbumin degradation, proteinuria and transfer to nonexchanging compartments.

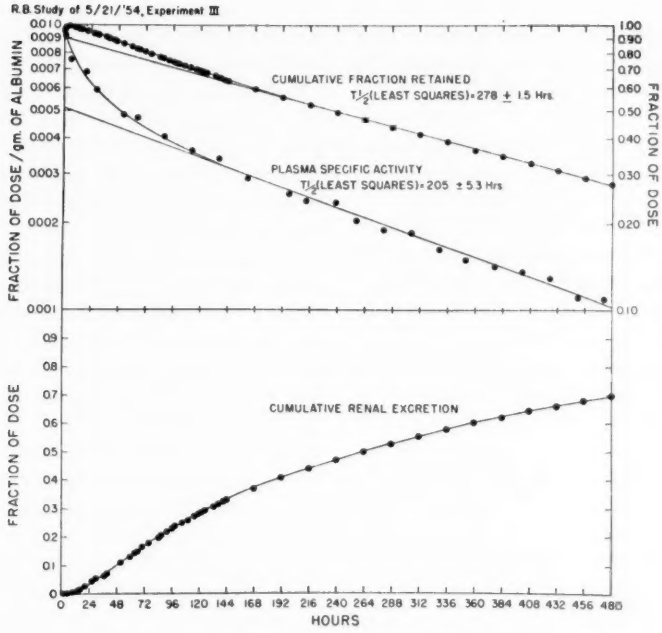

FIG. 1. TYPICAL PLASMA AND EXCRETION DATA OBTAINED IN TRACER STUDIES OF IODOALBUMIN METABOLISM

The coordinates of the upper two curves are semilogarithmic; those of the lower curve are linear.

b) $q_2(t) = A_{21}e^{-\alpha_1 t} + A_{22}e^{-\alpha_2 t} + A_{23}e^{-\alpha_3 t}$, where

$$\sum_i A_{2i} = 0$$

c) $q_3(t) = A_{31}e^{-\alpha_1 t} + A_{32}e^{-\alpha_2 t} + A_{33}e^{-\alpha_3 t}$, where

$$\sum_i A_{3i} = 0.$$

Equation *1* may be normalized to Equation *2a* by setting

3) $$A_{1i} = \frac{X_{1i}}{X_{11} + X_{12} + X_{13}}.$$

It follows, therefore, that experimental data obtained on the amount or specific activity of isotope in any one compartment or any linear combination of compartments should yield (within experimental error) the same set of slopes (α's) when analyzed as a sum of exponential terms.

Comparison of the retention curve and plasma specific activity curve in Figure 1 shows that both approach straight lines after about 120 hours. The terminal slopes of the lines are significantly different, however, with plasma having the greater slope. A similar significant discrepancy in terminal plasma and retention slopes measured over approximately the same time interval was encountered in all the tracer studies performed. From what has been said above, if the retention curve represented the sum of the exchanging albumin compartments, its final slope would have been the same as the final plasma slope. The slower slope of the retention curve suggests that it includes an additional compartment having negligible feedback to the exchanging system during the experiment. A similar discrepancy in slopes was noted in some but not all instances by Berson, Yalow, Schreiber and Post and was attributed to incomplete urine collections (7). Comparable findings are also evident in Myant's data in connection with studies of I^{131} gamma globulin (8). Further support for the loss of label from the exchanging system to a compartment other than urine comes from the fact that the asymptotic value of cumu-

lative urinary excretion was well below 100 per cent of the dose. Although measurements were not carried out long enough to determine the value of the asymptote experimentally, it can be computed as follows:

With $q_5(t)$ defined as the cumulative urinary excretion of label, a plot of the logarithm of the *daily* urinary excretion, $\frac{dq_5(t)}{dt}$, shows that its terminal slope (α_3) is virtually the same as the terminal slope of the plasma curve. If, at time t, the terminal slope of $\frac{dq_5(t)}{dt}$ has become established and if it is assumed that the terminal slope will remain constant indefinitely, one can compute the total expected excretion after time t_1:

$$4) \qquad \int_{t_1}^{\infty} \left[\frac{dq_5(t)}{dt}\right] dt = \left[\frac{dq_5(t_1)}{dt}\right] \frac{1}{\alpha_3},$$

where $\frac{dq_5(t_1)}{dt}$ is the rate of urinary excretion of label at time t_1. If the cumulative quantity of label in the urine at time t_1 is $q_5(t_1)$, the asymptotic value of urinary excretion (A_{55}) becomes:

$$5) \quad q_5(\infty) \equiv A_{55} = q_5(t_1) + \left[\frac{dq_5(t_1)}{dt}\right] \frac{1}{\alpha_3}.$$

In all the tracer studies performed, the value of A_{55} so computed was less than unity. The mean value obtained in 11 studies was 0.807. The difference of 0.193 must represent loss from the system into a site other than collected urine.

These findings indicated that even after a reasonable period for mixing, the label continues to leave the plasma at a faster rate than can be accounted for by collected urinary excretion and sampling. Among the more likely possibilities are the following:

1) Significant external loss of label by routes other than renal, or incomplete urinary collections. This seems unlikely in view of the demonstrated negligible fecal excretion and the recovery in urine of administered iodide I^{131} and diiodotyrosine I^{131} in amounts closely approaching 100 per cent of the administered dose. Label removed in sampling was quantitated and corrected for when it exceeded 1 per cent of the dose.

2) Entry of label into the thyroidal system with resultant delay in excretion. This is unlikely in view of the negligible fecal excretion and virtually quantitative urinary recovery of I^{131} administered as sodium iodide, verifying the adequacy of blockage of I^{131} uptake of any functioning thyroidal tissue present.

3) Loss of label to an extravascular compartment (exclusive of the thyroid) having a very slow turnover rate.

In this paper the third possibility is accepted

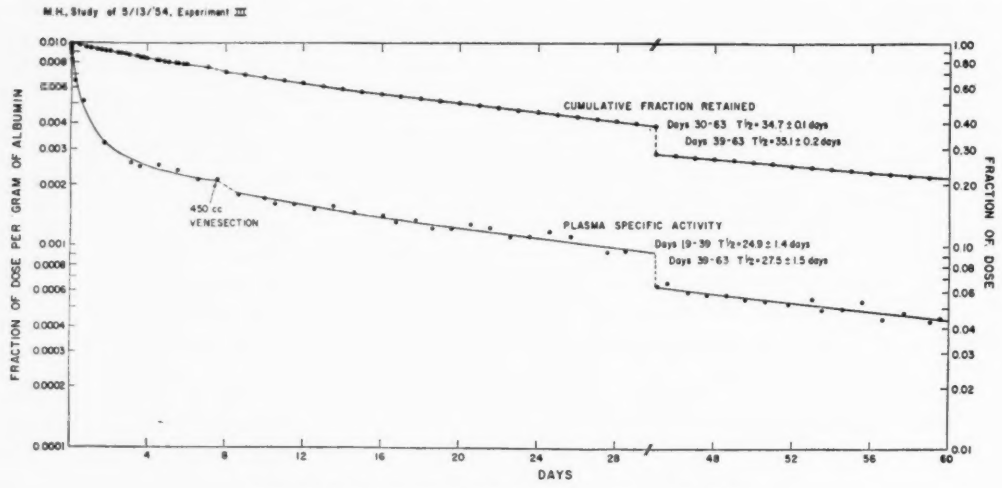

FIG. 2. METABOLISM OF I^{131} ALBUMIN IN PATIENT M. H.

Part of the plot has been omitted to facilitate presentation.

and the presence of an extravascular compartment showing negligible feedback of label to plasma during the course of the experiments is postulated. We wish to emphasize that the experimental evidence for the existence of this postulated Compartment 6 rests entirely on the fact that the calculated values for asymptotic urinary excretion were less than 100 per cent of the injected dose.

If an extravascular nonthyroidal compartment not readily in exchange with plasma exists, the question of its rate of turnover arises. After a sufficiently long time lapse, one would expect the final slopes of radioactivity in each compartment and the slope of their sum, the total label retention, to become equal to the slope of the plasma curve. Equality of plasma and retention slopes was not reached in studies carried out to 36 days [Experiment II, (2)]. For this reason fecal and urinary excretion of label, plasma specific activity and label removed in blood sampling were measured in the study of M. H., Experiment III, for 63 days.

The data are plotted in Figure 2. The breaks in the plasma specific activity and retention curves associated with the withdrawal and transfusion of blood on the eighth day are easily seen. The relative slowness with which the retention and plasma curves approach an apparent constant final slope might be explained by a nonhomogeneous population of injected iodoalbumin containing some elements more rapidly degraded than others or by the presence in the transfused blood of an agent capable of accelerating degradation.

In any event, the retention curve at about 30 days and the plasma curve at about 19 days closely approach an apparent constant final slope. The $T_{\frac{1}{2}}$ obtained by a least squares fit of the plasma curve from the nineteenth to thirty-ninth days is 24.9 ± 1.4 days, and from the thirty-ninth to sixty-third days, 27.5 ± 1.5 days. From the thirty-ninth to sixty-third days the $T_{\frac{1}{2}}$ of the retention curves is 35.1 ± 0.2 days. The difference in slope of the plasma and retention curves (thirty-ninth to sixty-third days) is highly significant statistically. The two curves show little tendency to become parallel. It is apparent that, in this patient, to achieve equality of slopes would require a period considerably longer than 63 days.

Tracer experiments of sufficient duration to allow quantitation of S_T (including the postulated slowly exchanging Compartment 6) appeared impractical. It seemed more reasonable to treat Compartment 6 analytically, not as an exchanging compartment, but as an internal, nonfeedback compartment.

Knowledge relative to the mechanism and site of iodoalbumin degradation is limited. Berson and Yalow (9) noted an apparent closer correlation between plasma radioactivity and renal excretion than between radioactivity in ascitic fluid or extravascular space and renal excretion, from which the major site of iodoalbumin degradation was inferred to be the plasma or some extravascular compartment equilibrating rapidly with plasma. The urine fractionation carried out in the case of G. S. (see "Methods") indicated that the excretion forms of label were principally iodide ion and diiodotyrosine.

It has been shown by Albert and Keating (10) and confirmed in this study that in the presence of adequate thyroid block, the radioactivity of labeled iodide ion or diiodotyrosine given intravenously is recovered virtually quantitatively in the urine. Similarly Skanse (11) reported a 72 hour urinary recovery of 98 per cent of I^{131} administered to thyroidectomized patients. This suggests that once the breakdown of iodoalbumin has progressed to the stage of iodide or iodinated tyrosines, the products of breakdown are quantitatively excreted in the urine. Degradation, subsequent to this stage, can reasonably be regarded as a nonfeedback process without reincorporation of iodine into protein.

The volume of distribution of iodide and its rate constant of excretion have been extensively studied. Representative figures from the literature give the volume of distribution of iodide as approximately 34 per cent of the body weight and the rate constant of iodide excretion as 0.072 per hour (12). Though diiodotyrosine is, for the most part, rapidly deiodinated when given intravenously and excreted principally as iodide, Albert and Keating (10) calculated the rate constant of excretion of diiodotyrosine *per se* as 0.013 per hour.

It is clear that once label is liberated from

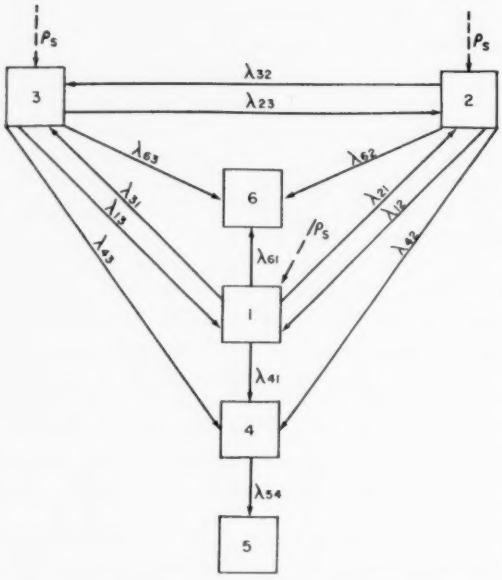

FIG. 3. A SIX-COMPARTMENT SYSTEM CONSISTING OF THREE EXCHANGING COMPARTMENTS (1, 2 AND 3) AND THREE ANCILLARY NONEXCHANGING COMPARTMENTS (4, 5 AND 6)

Compartment 1 (plasma) is the initially labeled and sampled compartment. Compartments 2 and 3 are, respectively, rapidly and slowly exchanging extravascular compartments. Compartment 6 is the postulated body compartment treated analytically as a nonexchanging compartment. Compartment 4 represents nonexcreted labeled degradation products. Compartment 5 is collected urine. The entry site of newly synthesized carrier is not restricted and is depicted (broken arrows) as being either of the exchanging compartments. The rate constants of transfer (the fractional turnover rates) and the steady state rate of carrier synthesis (ρ_s) appear beside the appropriately directed arrows.

iodoalbumin, it is not instantaneously excreted. Furthermore, the volumes of distribution of iodoalbumin and iodide are markedly different, comprising roughly 10 and 30 per cent, respectively, of the body weight. The excretion of liberated iodide will accordingly be modified by its transport into a larger volume of distribution and also by its rate constant of renal excretion. The excretion of noniodide degradation products will be similarly modified. These considerations led to the introduction into the model of a compartment (Compartment 4) to represent degradation products not yet excreted.

The model and its solution

The simplest general model that is compatible with the various considerations outlined above is shown in Figure 3. The meaning of the compartment numbers is given in and below the figure. The model consists of six compartments, three of which (1, 2 and 3) are exchanging compartments and three of which (4, 5 and 6) are nonexchanging compartments. The principal problem presented by this model is the determination of the configuration of the exchanging three-compartment system. Compartments 4, 5 and 6, the nonexchanging or ancillary compartments, must be considered, however, if solution of the configuration of the exchanging system is to be realized.

Most complete measurements of the system were made in the case of R. B., Experiment III. Tagged albumin which could reasonably be expected to be metabolically homogeneous because of previous tenure in a donor patient was introduced into plasma (Compartment 1) and repeated measurements of plasma specific activity and cumulative urinary excretion of label (Compartment 5) were made (Figure 1).

λ_{54} was assumed to be known on the basis of considerations to be discussed later. From knowledge of $q_5(t)$, $q_4(t)$ can be calculated from the relation:

$$\frac{dq_5(t)}{dt} = \lambda_{54} q_4(t)$$

and

$$6) \qquad q_4(t) = \frac{1}{\lambda_{54}} \frac{dq_5(t)}{dt}.$$

$\frac{dq_5(t)}{dt}$ can be obtained by either graphical or analytic differentiation of $q_5(t)$.

No measurements of the postulated Compartment 6 were made. By definition and from theoretical considerations (1) it follows that the asymptotic value of $q_6(t)$ must equal $1 - A_{55}$, the asymptotic value of $q_5(t)$, and that $q_6(t)$ must have the same exponential slopes (α_1, α_2 and α_3) as the plasma curve. From the available data, however, solution of the individual turnover rates λ_{61}, λ_{62} and λ_{63} is not possible. To pursue further the solution of the exchanging iodoalbumin system it is helpful to get an expression for the total radioactivity in Compart-

ments 2 and 3 as a function of time. This requires an expression for the radioactivity in Compartment 6 as a function of time. For mathematical simplicity it was assumed that the activity in Compartment 6 is proportional to the sum of the activity in Compartments 4 and 5 so that

$$q_6(t) = K[q_4(t) + q_5(t)]$$

and

$$q_5(\infty) + Kq_5(\infty) = 1,$$

where

$$K = \frac{1 - q_5(\infty)}{q_5(\infty)} = \frac{1 - A_{55}}{A_{55}}.$$

Although this assumption may result in completely erroneous values for λ_{61}, λ_{62} and λ_{63}, its effect on the turnover rates of the exchanging system is relatively small because $q_6(t)$ is small compared to $q_5(t)$.

From the data and the above assumptions regarding λ_{54} and Compartment 6, the total radioactivity in Compartments 2 and 3 can be calculated from the relation

$$7a) \quad q_2(t) + q_3(t) = 1 - [q_1(t) + q_4(t) + q_5(t) + q_6(t)] = 1 - \left[q_1(t) + \frac{q_4(t) + q_5(t)}{A_{55}}\right].$$

From Equations *2(b–c)* it follows that Equation *7a)* may be written:

$$b) \quad q_2(t) + q_3(t) = (A_{21} + A_{31})e^{-\alpha_1 t} + (A_{22} + A_{32})e^{-\alpha_2 t} + (A_{23} + A_{33})e^{-\alpha_3 t},$$

where

$$\sum_i (A_{2i} + A_{3i}) = 0.$$

The problem of the exchanging iodoalbumin system reduces to the general solution of a three-compartmental system when data on the compartment into which label is initially injected and the sum of the two other compartments is available. The considerations and solution that follow are based on the steady state analysis in Reference (1) and no effort will be made here to justify it.

The general three-compartment system (shown schematically in Figure 4) is characterized by nine independent fractional turnover rates, three for each compartment. Radioactivity-time measurements of any one compartment contain five independent constants: three α's and two A's. Only two of the three A's obtained from measurements of a sampled compartment are independent since the sum of the A's is fixed for each compartment (see Equation *2*). Two additional independent constants (two A's) are contained in the measurements of each additional compartment. Nine measured independent constants (six A's and three α's) corresponding to the nine independent fractional turnover rates (λ's) are required for a complete (unique) solution of the system. The data of R. B., Experiment III, contain seven independent constants: five from $q_1(t)$ (two A's and three α's) and two from $q_2(t) + q_3(t)$ (two A's). This means that $(9 - 7 =) 2$ independent variables are left undetermined. Consequently the data do not permit a unique solution of the system.[3] This means that there exist

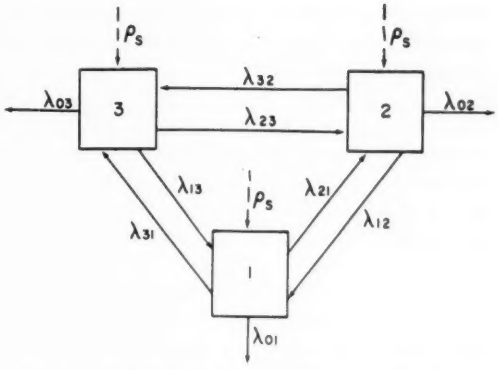

FIG. 4. THE GENERAL THREE-COMPARTMENT SYSTEM PROVIDING FOR TWO WAY EXCHANGE OF ANY ONE COMPARTMENT WITH EACH OF THE OTHER TWO AND FOR ENTRY OF NEW CARRIER INTO, AND EXCRETION OF CARRIER FROM, EACH OF THE COMPARTMENTS

Such a system is characterized by the nine independent fractional turnover rates shown beside each of the appropriately directed arrows. λ_{jj} is defined as the sum of the fractional turnover rates of compartment j; for example, $\lambda_{22} \equiv \lambda_{02} + \lambda_{12} + \lambda_{32}$.

[3] Subsequently we shall speak of a system to mean a given number of compartments without regard for the values of their turnover rates or the manner of their interconnection. A model will mean a system with a specified set of turnover rates that is mathematically consistent with the experimentally measured quantities. A physically realizable model will mean one in which all the turnover rates are positive or zero.

not one but an infinity of compatible models each of which is mathematically consistent with the experimental data.

Even though the number of models consistent with the data is infinite, the range of variation of each turnover rate for all possible compatible models may be quite restricted. It will now be shown how to obtain the range of variation of the turnover rates for all models and their values for a chosen model.

The procedures outlined follow from the treatment presented in Reference (1); they are, however, sufficiently complete so that they may be used without Reference (1). The original treatment starts with the conventional first order differential equations that describe the kinetics of isotopes. These are then transformed—using Laplace transform methods and matrix algebra—so that the solutions of turnover rates are expressible in simple algebraic equations and in terms of slopes and intercepts of the data expressed as a sum of exponential terms.

In the conventional solutions of model turnover rates using differential equations it is necessary that the data be complete to determine the model uniquely. When the data are incomplete some simplified model has to be assumed and then tested to see if it is compatible with the data. In special cases, certain turnover rates may be solved for directly from the data without assuming any model. There are no criteria, however, that can be applied to determine which models are or are not compatible with the data, or which turnover rates can or cannot be solved for directly. Furthermore, if one cannot think of a special model or solve for some turnover rates, much of the information contained in the data cannot be used.

The solution presented here is different from the above in that no assumptions as to any model have to be made. A general solution is obtained that represents the solution of all models. From the general solution, one can readily deduce which models are or are not possible without necessarily solving for the turnover rates of the models. Furthermore, the general solution permits the easy calculation of the turnover rates of any desired model and the determination of the range or degree of variation of any one turnover rate for all models.

There are various ways to obtain an expression for the general solution of the system. The method presented here was chosen because of its relative simplicity. It is broken down into two steps. The first step is to obtain a solution for the turnover rates of one model consistent with the data. The second step is to express the turnover rates of the general solution in terms of the solved model rates and two variables (x and y) that express the incompleteness of the data.

As already pointed out, the data are insufficient to determine a model uniquely. Hence, to solve for some compatible model, certain assumptions must be made. Here, again, there is a difference between the conventional differential equation methods and this method. In the former, assumptions are made about the model—*i.e.*, certain turnover rates are set to zero. Then one solves for the assumed model turnover rates and checks to see if the solution is compatible. In the method presented here, the assumptions are made about the missing data—*i.e.*, arbitrary values are assigned to them with the only restriction being that the initial conditions be satisfied. This may result in peculiar physical models in that some turnover rates may be negative. This, however, is of no significance in this method because mathematically, such a model is consistent with the data. The general solution merely uses the values of the solved model for an expression that includes all mathematically consistent models—those that are physically realizable and nonrealizable.

The assumptions made here about the missing data to yield the solution of *a* model are:

$$
\begin{array}{rrl}
 & q_2(t) = & (A_{22} + A_{32})e^{-\alpha_3 t} - (A_{22} + A_{32})e^{-\alpha_3 t} \\
+ & q_3(t) = (A_{21} + A_{31})e^{-\alpha_1 t} & - (A_{21} + A_{31})e^{-\alpha_3 t} \\
\hline
 & q_2(t) + q_3(t) = (A_{21} + A_{31})e^{-\alpha_1 t} + (A_{22} + A_{32})e^{-\alpha_3 t} - (A_{21} + A_{31} + A_{22} + A_{32})e^{-\alpha_3 t}.
\end{array}
$$

The expression for $q_2(t) + q_3(t)$ is the same as Equation *7b*.

The above assumptions are arbitrary. Several other ways to obtain the solution of a model are discussed in Reference (1).

Using these assumptions, the solution of the model turnover rates (this follows from any method of solution) are:

$$8a) \quad \lambda_{11}' = A_{11}\alpha_1 + A_{12}\alpha_2 + A_{13}\alpha_3$$

$$b) \quad \lambda_{12}' = \frac{A_{12}}{(A_{22} + A_{32})}[A_{11}(\alpha_1 - \alpha_2) - A_{13}(\alpha_2 - \alpha_3)]$$

$$c) \quad \lambda_{13}' = -\frac{A_{11}}{(A_{21} + A_{31})}[A_{12}(\alpha_1 - \alpha_2) + A_{13}(\alpha_1 - \alpha_3)]$$

$$d) \quad \lambda_{21}' = -(A_{22} + A_{32})(\alpha_2 - \alpha_3)$$

$$e) \quad \lambda_{22}' = (A_{11} + A_{13})\alpha_2 + A_{12}\alpha_3$$

$$f) \quad \lambda_{23}' = \frac{A_{11}(A_{22} + A_{32})}{(A_{21} + A_{31})}(\alpha_2 - \alpha_3)$$

$$g) \quad \lambda_{31}' = -(A_{21} + A_{31})(\alpha_1 - \alpha_3)$$

$$h) \quad \lambda_{32}' = \frac{(A_{21} + A_{31})(A_{12})}{(A_{22} + A_{32})}(\alpha_1 - \alpha_3)$$

$$i) \quad \lambda_{33}' = (A_{12} + A_{13})\alpha_1 + A_{11}\alpha_3$$

$$j) \quad \lambda_{01}' = (A_{11} + A_{21} + A_{31})\alpha_1 + (A_{12} + A_{22} + A_{32})\alpha_2 + (A_{13} + A_{23} + A_{33})\alpha_3.$$

The values of the A's and α's of these equations are the values of the A's and α's of Equations *2a* and *7b* that fit the experimental data.

The general solution can be obtained in terms of the values of the specific solution and the two variables (x and y) that represent the "missing" data. The equations were derived using a matrix mapping technique (1) whereby the given data are kept fixed for all the variations in the unknown data.[4] The general solution is:

$$9a) \quad \lambda_{11} = \lambda_{11}'$$

$$b) \quad \lambda_{12} = \frac{\lambda_{12}'(1 - y) - \lambda_{13}'(1 - x)}{x - y}$$

$$c) \quad \lambda_{13} = \frac{-\lambda_{12}'y + \lambda_{13}'x}{x - y}$$

$$d) \quad \lambda_{01} = \lambda_{01}'$$

$$e) \quad \lambda_{02} = \frac{\lambda_{02}'(1 - y) - \lambda_{03}'(1 - x)}{x - y}$$

$$f) \quad \lambda_{03} = \frac{-\lambda_{02}'y + \lambda_{03}'x}{x - y}$$

$$g) \quad \lambda_{21} = \lambda_{21}'x + \lambda_{31}'y$$

$$h) \quad \lambda_{22} = \frac{(\lambda_{22}'x - \lambda_{32}'y)(1 - y) + (\lambda_{23}'x - \lambda_{33}'y)(1 - x)}{x - y}$$

$$i) \quad \lambda_{23} = \frac{(\lambda_{22}'x - \lambda_{32}'y)y + (\lambda_{23}'x - \lambda_{33}'y)x}{x - y}$$

[4] In matrix notation the general solution of the $|\lambda|$ matrix may be expressed in terms of any specific solution $|\lambda'|$ matrix as

$$|\lambda| = |P|\,|\lambda'|\,|P^{-1}|,$$

where the matrix $|P|$ represents a mapping that preserves the given data $q_1(t)$ and $q_2(t) + q_3(t)$ and is of the form:

$$|P| = \begin{vmatrix} 1 & 0 & 0 \\ 0 & x & y \\ 0 & 1 - x & 1 - y \end{vmatrix}.$$

$$j) \quad \lambda_{31} = \lambda_{21}'(1 - x) + \lambda_{31}'(1 - y)$$

$$k) \quad \lambda_{32} = \frac{[-\lambda_{22}'(1 - x) + \lambda_{32}'(1 - y)](1 - y) - [\lambda_{23}'(1 - x) - \lambda_{33}'(1 - y)](1 - x)}{x - y}$$

$$l) \quad \lambda_{33} = \frac{-[\lambda_{22}'(1 - x) - \lambda_{32}'(1 - y)]y + [-\lambda_{23}'(1 - x) + \lambda_{33}'(1 - y)]x}{x - y}.$$

The primes refer to *any* compatible model that is already solved. Such a model will be called a "generating model" when it is used to generate all other mathematically compatible models from it, as indicated by Equations *9(a–l)*.

The substitution of any value of x and y into Equations *9(a–l)* results in the correct solution of some compatible model. On the other hand, the arbitrary assignment of some λ_{ij} to zero results in a constraint on the x's and y's. For each λ_{ij} a different constraint may result. Since there are only two variables and nine independent turnover rates, at most two turnover rates can in general be set equal to zero simultaneously. Thus, for example, a parallel model requires that $\lambda_{23} = \lambda_{32} = 0$. This results in two simultaneous equations in x and y, the solution of which yields a value of x and a value of y. Substitution of these values into Equations *9(a–l)* gives the values of the turnover rates for the parallel model.

The analysis presented is adequate when the data is in the required form. Certain difficulties that arise in practice will now be discussed with regard to the data of R. B., Experiment III.

In Experiment III, two corrections of the data as obtained involving Compartment 4 required consideration. *1)* The labeled plasma removed from M. H. and injected into R. B. was not dialyzed prior to injection to remove degradation products of iodoalbumin not yet excreted by the donor. A correction of the urinary excretion curve of R. B. for injected label not in the form of iodoalbumin was necessary. *2)* The plasma curve was obtained from counts on unfractionated plasma which contains some Compartment 4 activity. It is apparent that suitable corrections require that Compartment 4 activity as a function of time be known for the donor and recipient.

From theoretical considerations it can be shown *1)* that, for the system under consideration (see Figure 5), the cumulative urinary excretion curve is composed of four exponential components and a constant:

$$10) \quad q_5(t) = A_{51}e^{-\alpha_1 t} + A_{52}e^{-\alpha_2 t} + A_{53}e^{-\alpha_3 t} + A_{54}e^{-\lambda_{54} t} + A_{55},$$

where α_1, α_2 and α_3 are identical to the α's of the plasma curve and λ_{54} is the fractional turnover rate of Compartment 4. Theoretically, then, λ_{54} can be obtained directly by graphic analysis of $q_5(t)$. $\frac{dq_5(t)}{dt}$ can be obtained by graphic or analytical differentiation of $q_5(t)$, and $q_4(t)$ computed from Equation *6*.

From a practical standpoint, however, even with frequent early urine collections, resolution of the excretion curve into all of its components

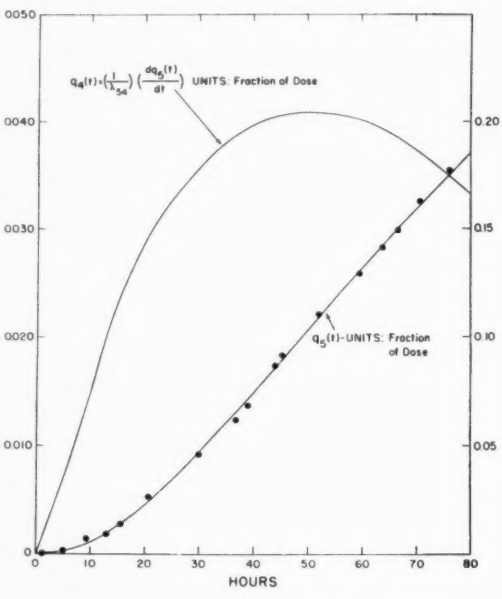

FIG. 5. GRAPHICAL DIFFERENTIATION OF $q_5(t)$

The left ordinate of the figure refers to $q_4(t)$; the right ordinate to $q_5(t)$.

within the restrictions imposed by the system was difficult and the reliability of the components other than the α_3 component and A_{55} was questionable. The values of α_1, α_2 and α_3 were considered to have been reliably determined from the plasma data and could be used as guides in resolving the components of the excretion curve. However, since the λ_{54} component does not appear in the plasma data (see Equation *2a*), it was desirable to have an independent measure of this constant.

In the fractionation study of urinary excretion forms of label performed on G. S., the label released by degradation of iodoalbumin appeared in the urine as 0.875 iodide and 0.125 diiodotyrosine (mean of two 24 hour collections). Studies by Albert and Keating (10) indicate that the rate constant of renal excretion of diiodotyrosine (K_{de}) is approximately 0.169 that of iodide (K_{ie}). Since λ_{54} is the mean rate constant of excretion of all forms of label from degraded iodoalbumin, it was approximated as a weighted average by the relationship:

$$11) \quad \lambda_{54} \cong 0.875\ K_{ie} + 0.125\ (0.169)\ K_{ie} = 0.896\ K_{ie}.$$

Following completion of the iodoalbumin experiment K_{ie} was determined for both the donor and recipient by independent NaI^{131} studies according to the method outlined by Keating, Power, Berkson and Haines (13) and λ_{54} for each was computed from the relationship in Equation *11*.

To estimate the volume of distribution of Compartment 4, iodide space was accepted as 0.34 of the body weight (12) and diiodotyrosine space as 0.53 of the body weight (10). This gave, as a weighted mean, a volume of distribution for Compartment 4 of 0.364 of the body weight.

In the case of the donor $q_4(t)$ was computed from Equation *6* for the time of withdrawal of labeled plasma. When the difference in distribution volumes of Compartments 4 and 1 was taken into account, it was calculated that approximately 0.018 of the label given to the recipient had not been in the form of iodoalbumin. The urinary excretion curve of the recipient was accordingly corrected for this quantity of label excreted at the rate of the value of λ_{54}. Similar calculations for 11 tracer studies in Experiments I, II and III indicated a mean maximum error of overestimation of plasma specific activity of 1.5 per cent from failure to fractionate plasma samples. In these studies this correction was disregarded. This computed noniodoalbumin label in plasma samples is similar to the values obtained by trichloroacetic acid precipitation (7).

The plasma specific activity, cumulative retention and cumulative urinary excretion curves for R. B., Experiment III, are shown in Figure 1. The exponential constants (α_i) and the coefficients (X_{1i}) of the plasma curve were determined by standard methods of graphic analysis (3). The coefficients X_{1i} were normalized by Equation *3*. λ_{54} was computed from Equation *11*. $\frac{dq_5(t)}{dt}$ was obtained by graphical differentiation of $q_5(t)$ and $q_4(t)$ was computed from Equation *6* (see Figure 5). Finally, $q_2(t) + q_3(t)$ was computed from Equation *7a*. The values of the various constants were:

A_{11}: 0.146	α_1: 0.180
A_{12}: 0.326	α_2: 0.0301
A_{13}: 0.528	α_3: 0.00339
	λ_{54}: 0.0739

It was now desirable to resolve the curve of $q_2(t) + q_3(t)$ into its exponential components so as to obtain an expression for $q_2(t) + q_3(t)$ having the form of Equation *7b*. The plot of $q_2(t) + q_3(t)$ obtained from the experimental data is shown in Figure 6 as Curve A. In fitting exponential components to this curve the following conditions were preserved:

a) $(A_{21} + A_{31}) + (A_{22} + A_{32}) + (A_{23} + A_{33}) = 0$ (see Equation *2b, c*)

b) that the exponential constants be the same as those of the plasma curve (see Equation *2a–c*).

Rather than reiterate the various approaches used in fitting the curve, it is perhaps more instructive to discuss Curve B, which represents the sum of the components finally accepted as best fitting the curve. Inspection of the figure shows that the fit of the α_3 component with $(A_{23} + A_{33}) = 0.431$ is exact. Acceptance of the value of $(A_{23} + A_{33})$ fixes the sum of $(A_{21} + A_{31}) + (A_{22} + A_{32})$ from condition *a*) above. Now

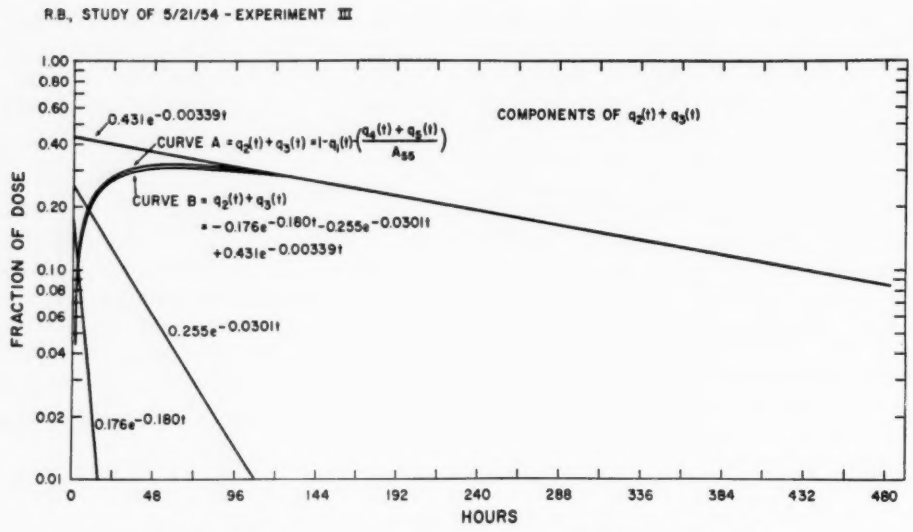

FIG. 6. RESOLUTION OF THE COMPONENTS OF THE CURVE $q_2(t) + q_3(t)$

Curve A is readily calculable from the experimental data. Curve B represents the sum of the "best fit" components obtained by procedures described in the text.

inspection of Equations *8j* and *9d* shows that λ_{01} is uniquely determined from the measured or readily calculable quantities $q_1(t)$ and $q_2(t) + q_3(t)$ and is therefore independent of the choice of model. It follows that $\lambda_{01}' = \lambda_{01} \geqq 0$ if a physically realizable model exists. Setting Equation $8j \geqq 0$, and substituting known values for A_{1i} and α_i and 0.431 for $(A_{23} + A_{33})$ in condition *a*), and solving simultaneously one obtains:

$$(A_{22} + A_{32}) \leqq -0.255$$
$$(A_{21} + A_{31}) \geqq -0.176.$$

Curve B represents the sum of the fitted components for $\lambda_{01} = 0$: $(A_{21} + A_{31}) = -0.176$ $(A_{22} + A_{32}) = -0.255$, and $(A_{23} + A_{33}) = 0.431$.

TABLE I

*Solution of data of R. B., Experiment III. Fractional turnover rates for four compatible and physically realizable models and a compatible "generating" model**

	Model designation				
	$\lambda_{23} = \lambda_{32} = 0$ x = −0.27916 y = 0.94621	$\lambda_{21} = \lambda_{31} = 0$ x = −0.38466 y = 1.3038	$\lambda_{31} = \lambda_{03} = 0$ x = −0.35422 y = 1.2971	$\lambda_{21} = \lambda_{03} = 0$ x = −0.25862 y = 0.94704	"Generating" model x = 1 y = 0
λ_{11}	0.03788†	0.03788	0.03788	0.03788	0.03788
λ_{12}	0.1236	0.09495†	0.09495	0.1236†	−0.009942
λ_{13}	0.01920	0.01920†	0.01749†	0.01749	0.1180
λ_{01}	0†	0	0	0	0
λ_{02}	0.03230	0.02359†	0.02359	0.03230†	−0.008355
λ_{03}	0.0005184	0.0005184†	0†	0	0.03059
λ_{21}	0.02749†	0.03788†	0.03788	0.02766	0.006816
λ_{22}	0.1559	0.1559	0.1551†	0.1559†	0.02139
λ_{23}	0†	0	0.003052†	0.002228	0.005657
λ_{31}	0.01039†	0†	0	0.01022	0.03107
λ_{32}	0†	0.03734†	0.03652	0	0.03969
λ_{33}	0.01972†	0.01973	0.02055†	0.01972	0.1542

* λ values are presented to four figures to preserve the internal consistency of the model solutions and are not considered to reflect experimental precision.

† An upper or lower limiting value of the respective turnover rate for all physically realizable models.

To avoid a negative value of λ_{01}, $(A_{22} + A_{32})$ and $(A_{21} + A_{31})$ may vary only as shown above. Inspection of Figure 6 shows that Curve B fits the experimental Curve A at the beginning and end of the curves but that the discrepancy occurs in a region where the contribution of the α_1 component to the curve is negligible. From the figure it is clear that if $(A_{22} + A_{32})$ and $(A_{21} + A_{31})$ are allowed to take on values consistent with a positive value of λ_{01} as indicated above, the discrepancy between Curves A and B will increase. It was concluded that the component values of Curve B represented the best obtainable fit of the data that would avoid a negative value of λ_{01}.

These values of the coefficients of $q_2(t) + q_3(t)$ and the values previously given for A_{1i}, α_i and λ_{54}, when substituted in Equations *8(a–j)*, yielded the solution of the generating model shown in Table I. It can be seen that this generating model has two negative fractional turnover rates. This does not matter since it is merely a mathematically compatible model. All other compatible models are expressed in terms of the variables x and y and the turnover rates of the generating model [see Equations *9(a–l)*]. The values of x and y that yield physically realizable models will be determined later.

Equations *9(a–l)* indicate that if any turnover rate that is a function of x and y is set equal to zero, a constraint on x and y results. For ex-

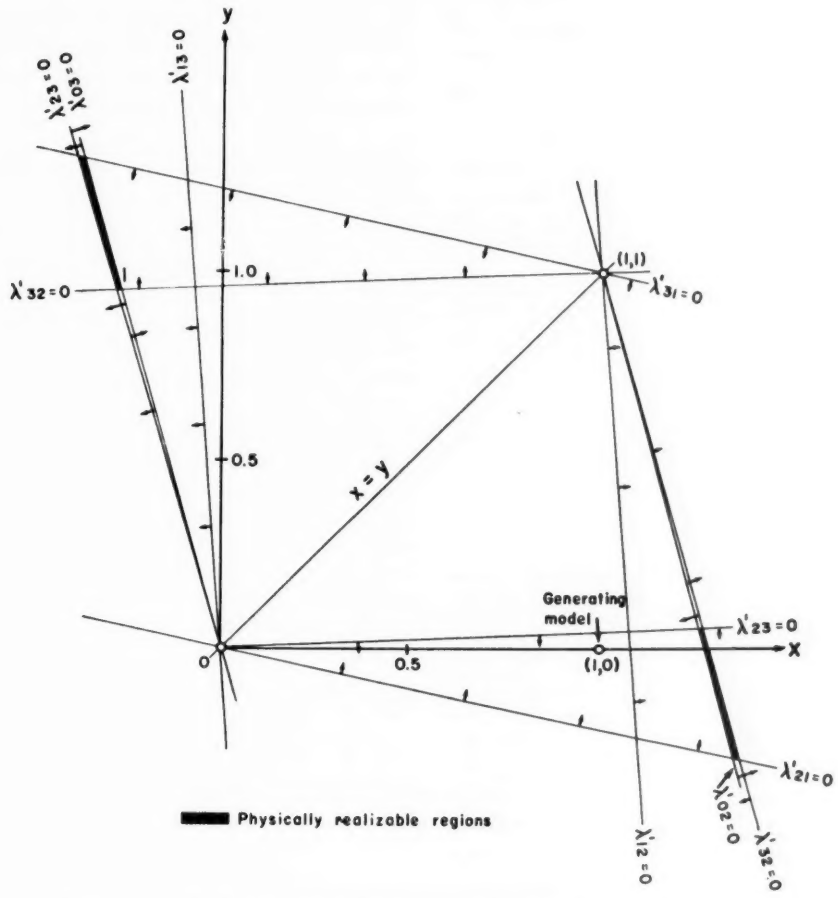

FIG. 7. A PLOT OF THE LINEAR RELATIONSHIPS BETWEEN X AND Y OBTAINED BY SETTING THE RESPECTIVE TURNOVER RATES TO ZERO

ample, if

$$\lambda_{12} = \frac{\lambda_{12}'(1 - y) - \lambda_{13}'(1 - x)}{x - y} = 0$$

$$\frac{1 - y}{1 - x} = \frac{\lambda_{13}'}{\lambda_{12}'}$$

and if

$$\lambda_{03} = 0, \quad \frac{y}{x} = \frac{\lambda_{03}'}{\lambda_{12}'}, \quad \text{etc.}$$

Substituting the values of the λ_{ij}'s of the generating model into each of the possible constraints of x and y, one obtains the following:

for *a*) $\lambda_{21} = 0, \quad \frac{y}{x} = -0.219$

b) $\lambda_{31} = 0, \quad \frac{1 - y}{1 - x} = -0.219$

c) $\lambda_{01} \neq 0$, no constraint since λ_{01} is constant for all models

d) $\lambda_{12} = 0, \quad \frac{1 - y}{1 - x} = -11.9$

e) $\lambda_{32} = 0, \quad \frac{1 - y}{1 - x} = -3.39, 0.0421$

f) $\lambda_{02} = 0, \quad \frac{1 - y}{1 - x} = -3.66$

g) $\lambda_{13} = 0, \quad \frac{y}{x} = -11.9$

h) $\lambda_{23} = 0, \quad \frac{y}{x} = 0.0421, -3.39$

i) $\lambda_{03} = 0, \quad \frac{y}{x} = -3.66.$

Each of these constraints results in a linear relation between x and y and is plotted as a straight line in Figure 7. It follows that if the plotted lines yield values of zero for the corresponding turnover rates, that on one side of the line the values will be positive and on the other side negative. The positive and negative sides can be determined by carefully checking each constraint. The arrows in Figure 7 indicate the direction where the positive values of the λ's must lie. Examination of all the arrows shows that only in the shaded regions can all the turnover rates be zero or positive. These regions therefore correspond to all the physically realizable models. Any value of x and y in these regions substituted in Equations *9*(*a–l*) yields a mathematically compatible and physically realizable solution.

Figure 7 shows two shaded regions where physically realizable models can lie. The two regions are actually symmetric with respect to each other, in that Compartments 2 and 3 are interchanged. This results from the fact that the data give the sum of $q_2(t) + q_3(t)$ and the solution does not distinguish between the two. For the purposes of this discussion we shall neglect the region in the lower right side and speak of the other one only. The position of the generating model is also shown in the plot.

Figure 7 shows that only the following turnover rates (in addition to λ_{01}) may be zero: λ_{23}, λ_{03}, λ_{31} and λ_{32}, and only the following combinations may be zero simultaneously:

a) $\lambda_{32} = \lambda_{23} = 0$ *c*) $\lambda_{31} = \lambda_{03} = 0$

b) $\lambda_{23} = \lambda_{31} = 0$ *d*) $\lambda_{32} = \lambda_{03} = 0.$

The values of x and y for each of these four mathematically compatible and physically realizable "simplest" models and the values of their turnover rates obtained from Equations *9*(*a–l*) are shown in Table I. They are termed "simplest" because they require the minimum number of interconnections.

Complete knowledge of all the turnover rates in the system is not sufficient to determine the compartment sizes; the site of entry and the rate of synthesis of newly synthesized carrier must also be known. Assuming each of the three exchanging compartments in turn to be the sole site of entry of newly synthesized carrier into the system we can write, for entry into Compartment 1:

12a) $$S_1' = \frac{\lambda_{22}'\lambda_{33}' - \lambda_{23}'\lambda_{32}'}{\alpha_1\alpha_2\alpha_3}\rho_s'$$

b) $$S_2' = \frac{\lambda_{21}'\lambda_{33}' + \lambda_{31}'\lambda_{23}'}{\alpha_1\alpha_2\alpha_3}\rho_s'$$

c) $$S_3' = \frac{\lambda_{21}'\lambda_{32}' + \lambda_{31}'\lambda_{22}'}{\alpha_1\alpha_2\alpha_3}\rho_s',$$

where ρ_s' is the rate of entry of newly synthesized carrier into the system per unit time. For entry

into Compartment 2:

$$13a) \qquad S_1' = \frac{\lambda_{12}'\lambda_{33}' + \lambda_{13}'\lambda_{32}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'$$

$$b) \qquad S_2' = \frac{\lambda_{11}'\lambda_{33}' - \lambda_{13}'\lambda_{31}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'$$

$$c) \qquad S_3' = \frac{\lambda_{11}'\lambda_{32}' + \lambda_{12}'\lambda_{31}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'.$$

For entry into Compartment 3:

$$14a) \qquad S_1' = \frac{\lambda_{12}'\lambda_{23}' + \lambda_{22}'\lambda_{13}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'$$

$$b) \qquad S_2' = \frac{\lambda_{11}'\lambda_{23}' + \lambda_{21}'\lambda_{13}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'$$

$$c) \qquad S_3' = \frac{\lambda_{11}'\lambda_{22}' - \lambda_{12}'\lambda_{21}'}{\alpha_1\alpha_2\alpha_3}\,\rho_s'.$$

Now S_1 is independent of the model chosen:

$$15) \qquad S_1 = S_1' = \text{constant} = \frac{1}{\sum_i X_{1i}}.$$

Accordingly for any assumed or known site of entry, S_1' may be obtained from Equation *15* and ρ_s', S_2' and S_3' computed by appropriate substitution in Equation *12*, *13* or *14*. Furthermore, for entry into Compartment 1, the solution for all models in terms of x and y is:

$$16a) \qquad S_2 = x\,S_2' + y\,S_3'$$

$$b) \qquad S_3 = (1 - x)\,S_2' + (1 - y)\,S_3'.$$

From Equation *16* it follows that $S_2 + S_3 = S_2' + S_3'$ = constant for all models with Compartment 1 as the site of entry and $S_1 + S_2 + S_3 = S_1' + S_2' + S_3'$ = constant for all such models. The values of S_j and ρ_s for the four simplest models are tabulated in Table II, assuming in turn each of the exchanging compartments to be the site of entry of newly synthesized albumin into the system. The considerable dependence of ρ_s and S_j on the site of entry is apparent.

The amount of carrier albumin that flows to any compartment i from any other compartment j may be computed from the relation

$$17) \qquad \rho_{ij} = \lambda_{ij}S_j.$$

Since all physically realizable models are expressed as a region in the x − y plane, the range of variation of each turnover rate for all permissible models can be obtained by investigating

TABLE II

Solution of data of R. B., Experiment III. The effect of the site of entry of newly synthesized carrier into the exchanging system on S_j, ρ_s, and λ_{OT} for four compatible, physically realizable models and a compatible "generating" model *

Site of carrier entry		Model designation				
		$\lambda_{23} = \lambda_{32} = 0$	$\lambda_{23} = \lambda_{21} = 0$	$\lambda_{31} = \lambda_{03} = 0$	$\lambda_{32} = \lambda_{02} = 0$	"Generating" model
Compartment 1	S_1	103.09	103.09	103.09	103.09	103.09
	S_2	18.19	25.06	26.10	19.06	41.14
	S_3	54.31	47.44	46.40	53.44	31.36
	$S_2 + S_3$	72.50	72.50	72.50	72.50	72.50
	$S_1 + S_2 + S_3$	175.59	175.59	175.59	175.59	175.59
	ρ_s	0.6159	0.6159	0.6159	0.6159	0.6159
	λ_{OT}†	0.003508	0.003508	0.003508	0.003508	0.003508
Compartment 2	S_1	103.09	103.09	103.09	103.09	103.09
	S_2	23.17	29.75	30.98	24.04	71.29
	S_3	54.31	56.31	55.08	53.44	39.12
	$S_2 + S_3$	77.48	86.06	86.06	77.48	110.40
	$S_1 + S_2 + S_3$	180.57	189.15	189.15	180.57	213.49
	ρ_s	0.7769	0.7311	0.7311	0.7769	0.6014
	λ_{OT}†	0.004303	0.003865	0.003865	0.004302	0.002817
Compartment 3	S_1	103.09	103.09	103.09	103.09	103.09
	S_2	18.18	25.05	26.73	19.51	42.55
	S_3	86.39	79.52	78.19	85.40	36.69
	$S_2 + S_3$	104.57	104.57	104.92	104.91	79.24
	$S_1 + S_2 + S_3$	207.66	207.66	208.01	208.00	182.33
	ρ_s	0.6328	0.6328	0.6307	0.6307	0.7675
	λ_{OT}†	0.003047	0.003047	0.003032	0.003032	0.004209

* Solution values are presented to an arbitrary four or five figures to preserve the internal consistency of the model solutions and are not considered to reflect experimental precision.

† Compare values to $\alpha_3 = 0.00339$.

their variation over the entire region. The maximum and minimum values of each turnover rate for all physically realizable models are indicated in Table I.

The specific activity of any compartment may be defined as the ratio of labeled to carrier albumin and is a function of time, the compartment sizes and the turnover rates. It is frequently desirable to express the ratio between the specific activities of two or more compartments for large values of time. This can be expressed as

$$\frac{x_i}{x_j} = \frac{A_{in}S_j}{A_{jn}S_i} \text{ for } t \gg 0.$$

In general S_j/S_i is dependent on the model and the rate of synthesis and site of entry of carrier. If it is assumed that newly synthesized carrier enters Compartment 1 only, then

$$18a) \quad \frac{x_2}{x_1} = \left[\frac{\lambda_{22}\lambda_{33} - \lambda_{23}\lambda_{32}}{(\lambda_{22} - \alpha_3)(\lambda_{33} - \alpha_3) - \lambda_{23}\lambda_{32}} \right] \times \left[\frac{\lambda_{21}(\lambda_{33} - \alpha_3) + \lambda_{31}\lambda_{23}}{\lambda_{21}\lambda_{33} + \lambda_{31}\lambda_{23}} \right] \text{ for } t \gg 0.$$

Similarly,

$$18b) \quad \frac{x_3}{x_1} = \left[\frac{\lambda_{22}\lambda_{33} - \lambda_{23}\lambda_{32}}{(\lambda_{22} - \alpha_3)(\lambda_{33} - \alpha_3) - \lambda_{23}\lambda_{32}} \right] \times \left[\frac{\lambda_{21}\lambda_{32} + \lambda_{31}(\lambda_{22} - \alpha_3)}{\lambda_{21}\lambda_{32} + \lambda_{31}\lambda_{22}} \right] \text{ for } t \gg 0.$$

It can be shown that the first bracketted term on the right hand side of these equations is independent of the model chosen. The second term, however, does depend on the model chosen. For the special case of a parallel model ($\lambda_{23} = \lambda_{32} = 0$) these equations reduce to

$$18c) \quad \frac{x_2}{x_1} = \frac{\lambda_{22}}{\lambda_{22} - \alpha_3} \text{ for } t \gg 0$$

$$d) \quad \frac{x_3}{x_1} = \frac{\lambda_{33}}{\lambda_{33} - \alpha_3} \text{ for } t \gg 0.$$

In this connection, the data of C. C. shown in Figure 5 of Reference (2) are of considerable interest. Shortly after the beginning of treatment with L-triiodothyronine there is an apparent rise in plasma specific activity which would require entry into the plasma of labeled material from a compartment having a specific activity greater than that of plasma.

From Figure 7 it is apparent that the parallel model ($\lambda_{23} = \lambda_{32} = 0$) is a physically realizable model but that the series model is excluded (the intersection of the lines $\lambda_{31}' = 0$ and $\lambda_{13}' = 0$ is outside the shaded area). From Tables I and II it can be seen from the solutions of the four simplest limiting models that in general the exchange of albumin (grams per hour) across the boundary between Compartments 2 and 3 is smaller than that across the boundaries between Compartments 1 and 3 or 1 and 2. These facts and certain physiological considerations lead us to favor the parallel model as the one most closely resembling the biological system. Further application of the parallel model solution will be considered elsewhere (2). Once a model has been chosen, it is of considerable interest to construct curves of radioactivity and specific activity as a function of time for each of the compartments as this serves to clarify certain implications of the solution. For the parallel model with the ancillary Compartments 4, 5 and 6 having the configuration shown in Figure 3, the theoretical equations for radioactivity as a function of time in Compartments 1, 2, 3 and 5 are given above (Equations *2* and *10*). The corresponding equations for Compartments 4 and 6 are:

$$19a) \quad q_4(t) = A_{41e}^{-\alpha_1 t} + A_{42e}^{-\alpha_2 t} + A_{43e}^{-\alpha_3 t} + A_{44e}^{-\lambda_{54} t}$$

where

$$\sum_i A_{4i} = 0$$

$$b) \quad q_6(t) = A_{61e}^{-\alpha_1 t} + A_{62e}^{-\alpha_2 t} + A_{63e}^{-\alpha_3 t} + A_{64}$$

where

$$\sum_i A_{6i} = 0.$$

The following additional relationships, together with the solution given in Table I, may be used to compute the coefficients A_{ji} for all nonsampled compartments and for experimental compartment curves not completely resolved into exponential components.

$$20a) \quad A_{2i} = \frac{\lambda_{21}}{\lambda_{22} - \alpha_i} A_{1i}$$

$$b) \quad A_{3i} = \frac{\lambda_{31}}{\lambda_{33} - \alpha_i} A_{1i}$$

$$c) \quad \lambda_{6j} = \lambda_{0j}(1 - A_{55}) = \lambda_{0j}A_{64}$$

$$d) \quad \lambda_{4j} = \lambda_{0j}A_{55}$$

$$e) \quad A_{4i} = \frac{A_{1i}\lambda_{41} + A_{2i}\lambda_{42} + A_{3i}\lambda_{43}}{\lambda_{54} - \alpha_i} ;$$

$$A_{44} = -\sum_{i=1}^{3} A_{4i}$$

$$f) \quad A_{5i} = -\frac{A_{4i}\lambda_{54}}{\alpha_i} ; \quad A_{54} = -A_{44}$$

$$g) \quad A_{6i} = (A_{4i} + A_{5i})\frac{A_{64}}{A_{55}} .$$

By substitution of the coefficients A_{ji} in Equations *2*, *10* and *19*, radioactivity-time curves, and, with appropriate normalization, specific activity-time curves for all compartments may be constructed. These plots for the tracer study under discussion are shown in Figure 8. Compartment 1 is assumed to be the site of entry of newly synthesized carrier. The inequality of specific activities in the exchanging compartments for

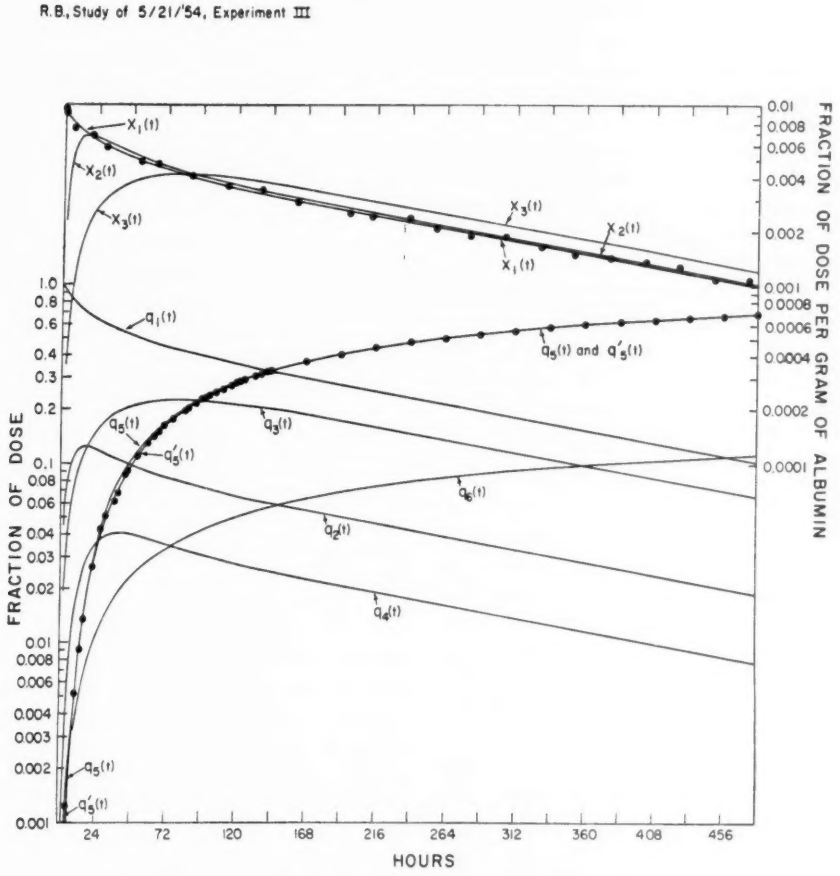

FIG. 8. GRAPHICAL REPRESENTATION OF THE OPEN PARALLEL SOLUTION

"q" curves are plotted with reference to the left ordinate, "x" curves with reference to the right ordinate. The curve labeled $q_5'(t)$ is the experimental urinary excretion curve. The curve labeled $q_5(t)$ represents the theoretical excretion curve for the model $\lambda_{23} = \lambda_{32} = 0$ with Compartment 1 assumed to be the site of carrier entry. The solution parameters for this model appear in Tables I and II. The points on curve $x_1(t)$ are experimental. The curve as drawn is that reconstructed from the component values of $q_1(t)$ given in the text with appropriate normalization.

large values of time is apparent. The degree to which the plotted experimental and theoretical excretion curves coincide is a measure of the closeness of fit of the experimental data to this model.

DISCUSSION

The analysis presented in this paper hinges predominantly on a system of three exchanging compartments. The additional compartments introduced (Compartments 4, 5 and 6) must be considered for quantitation of tracer within the exchanging system but do not influence the choice of the number of exchanging compartments. The latter is determined from the analysis of the plasma data alone.

Two independent sets of quantities may be distinguished in the general solution presented. One set consists of the fractional turnover rates of the compartments and these are determined from the tracer data alone. The second set consists of the amounts of carrier in each compartment and the rate of synthesis of carrier and its site of entry into the system. Specific activities are functions of both the tracer and carrier quantities.

Certain features of the solution presented (applicable to any three-compartment system for which data on Compartment 1 and the sum of Compartments 2 and 3 are available) are worth mentioning. The number of models which are mathematically compatible with the data and physically realizable is theoretically infinite, because any value of x and y in the shaded region of Figure 7 yields such a model. Despite this, a great deal can be said about the system. First, if one wishes to limit the solution to models that have the smallest number of interconnections, it is clear from Figure 7 that only four such models exist, the parallel model being one of these. The figure also indicates which models are not physically realizable. As noted above, the series model is excluded.

From Figure 7 it can be seen that (in addition to λ_{01}) at most four fractional turnover rates (λ_{23}, λ_{31}, λ_{03} and λ_{32}) may assume a value of zero, and that at most two of these may be zero in any single realizable model. Despite the infinity of realizable models, the values of the individual turnover rates may either be unique or vary over a limited range. Thus λ_{11}, λ_{01}, $\lambda_{21} + \lambda_{31}$ and $\lambda_{22} + \lambda_{33}$ are constant for all models. λ_{22}, λ_{33} and λ_{03} vary over very small ranges, whereas others show greater variation. Of particular interest is the fact that λ_{02} is greater than zero in all realizable models and is consistently much larger than λ_{03}. This suggests that the rapidly exchanging extravascular compartment is the principal site of iodoalbumin degradation, a conclusion which is independent of the choice of model.

The values for the compartment sizes and rate of synthesis depend in part on the assumption of the site of entry of newly synthesized carrier. Assuming Compartment 1 to be the site of entry the solution for S_1 and for $S_2 + S_3$ and consequently for $S_1 + S_2 + S_3$ is the same for all mathematically compatible models. Under the same conditions it is evident from Table II that ρ_s is constant for all compatible models. For each different assumption concerning the site of entry, however, the values of $S_2 + S_3$, $S_1 + S_2 + S_3$, and of ρ_s may be quite different. This is shown in Table II where solution values are given for three different sites of entry.

It is noteworthy that all the tracer data collected have been completely utilized in the solution presented. It is impossible therefore to reduce the uncertainty in the solution any further, regardless of the mathematical approach used, unless additional assumptions are made or new independent data be obtained.

In certain situations it may not be practical to apply routinely to tracer data a mathematical solution as general as the one presented here. One of the principal values of such a general approach, however, is that it formulates relationships inherent in the arbitrary assumption of model configurations which are sometimes not readily apparent. In addition it provides a way to evaluate the error in approximation methods and thereby serves to aid in the delineation of the conditions under which they are, or are not, acceptable.

In this connection it is of some interest to compare the values of certain parameters of the system obtained by the solution presented with the values obtained by approximation methods commonly employed. The "intercept" method is frequently used to compute total exchange-

able carrier, S_T, from the equation:

21a) $S_T = S_1/A_{1n}$ or the equivalent expression

b) $S_T = 1/X_{1n}$,

where S_1 is the size of the sampled compartment and A_{1n} and X_{1n} are the ordinate intercepts of the final exponential components of the radioactivity or specific activity, respectively, of the sampled compartment. Here X_{1n} is assumed to represent what the specific activity of the system as a whole would be at zero time if all the administered label were instantaneously and uniformly distributed throughout the system. Two sources of error are evident. *1)* x_1 for $t \gg 0$ does not represent the mean specific activity of the system as a whole (see Equation *18*). For the parallel model with entry of new carrier into Compartment 1, for $t \gg 0$, the value of $\frac{x_2}{x_1}$ was 1.02, $\frac{x_3}{x_1}$ was 1.21, and $\frac{q_1 + q_2 + q_3}{S_1 + S_2 + S_3} \Big/ x_1$ was 1.07. In general, the underestimation of the mean "equilibrium" specific activity of the system as a whole, if it be equated to that of the initially labeled compartment, leads to an overestimation of S_T by Equation *21*. Other things being equal, the magnitude of the error is dependent on the relative magnitude of α_3 compared to λ_{22} and λ_{33}. *2)* The intercept method assumes that the fractional rate of change of specific activity or radioactivity of the sampled compartment due to excretion of label from the system is constant throughout the experiment, and that $\frac{d[q_1(t)+q_2(t)+q_3(t)]}{dt} \Big/ [q_1(t)+q_2(t)+q_3(t)] = \alpha_n = \text{constant}$, where n refers to the final component of the sampled compartment. This in turn assumes that $\lambda_{02} = \lambda_{03} = \lambda_{01} = \lambda_{0T}$. Where inequalities of the rates of excretion from various compartments occur, this may lead to a greater or smaller fractional rate of excretion of total label in the early, as compared to the late, phases of the experiment, which will be reflected in smaller, or larger values, respectively, of A_{1n} and X_{1n}. In general, where the fractional rate of excretion of total label in the early phase of the experiment exceeds that in the late phase, the effect is reflected in a smaller value of X_{1n}, which increases the estimation of S_T by Equation *21*. The converse also applies.

For the tracer data presented here, Equation *21* gave a value for S_T of 195.3 Gm. as compared to 175.6 Gm. by three-compartment solution assuming new carrier entry into Compartment 1. Experimental verification of some of the above considerations is found in the data of Gitlin, Janeway and Farr (14) who, in labeled iodoprotein studies in nephrosis, encountered measured values of edema fluid specific activity many times that of the plasma specific activity for large values of time and unreasonably large values of S_T by the intercept method.

When total label in the exchanging system (q_T) can be computed from excretion data (with appropriate correction for ancillary compartments such as Compartments 4 and 6) S_T is sometimes estimated by the "dilution" equation:

22) $$S_T = \frac{q_T}{x_1} \text{ for } t \gg 0.$$

The error in this method depends on the degree to which x_1 differs from the mean specific activity of the system as a whole as discussed above.

In biological tracer work, the parameters of the systems studied which commonly receive greatest attention are S_T, ρ_s and λ_{0T}. In a three-compartment open system,

$$\lambda_{0T} \equiv \frac{S_1\lambda_{01} + S_2\lambda_{02} + S_3\lambda_{03}}{S_1 + S_2 + S_3}.$$

These three quantities are interrelated in the steady state:

23) $$\rho_s = S_T\lambda_{0T}.$$

The sources and magnitudes of the errors involved in estimating S_T by "intercept" and "dilution" methods have been discussed above. The commonly employed "slope" method equates λ_{0T} to α_n where n, as before, refers to the final exponential component of the sampled compartment and takes on a value of 3 for a system of three exchanging compartments. Examination of Table II where values of λ_{0T} are given for the four limiting models and the generating model shows that α_3 may underestimate or overestimate λ_{0T} depending on the choice of model configuration and site of new carrier entry. For assumed entry into Compartment 1, λ_{0T} is constant and greater than α_3.

The turnover rates of body proteins are of

considerable theoretical importance. The difficulties encountered in attempts to measure a plasma protein turnover rate have been discussed at length by Luck (15) and will not be reiterated here. A faster turnover rate for serum proteins determined by I^{131} labeling as compared to biosynthetic labels with C^{14} or S^{35} has been reported by several workers (16, 17). By jet iodination, however, McFarlane (18) has reported an I^{131} labeled albumin which appears to behave in an identical fashion to albumin biosynthetically labeled with C^{14}. Furthermore, Berson, Yalow, Schreiber and Post (7) have described multiple components apparently metabolized at different rates in the same preparation of iodoalbumin. Yalow and Berson have also reported evidence that I^{131} albumin of high specific activity may be damaged by self-irradiation to such an extent that its apparent rate of degradation is accelerated (19). Furthermore, it is apparently not clear, where discrepancies between the turnover rates determined by the different types of labels have been encountered, to what extent the discrepancy is attributable to reutilization of the C^{14} or S^{35} label. We are aware of the questionable validity of iodoalbumin as a label for endogenous albumin. Further discussion of this subject is not pertinent here. It must be emphasized that the derived rates of transport, degradation and "sequestration" are valid only insofar as the biological behavior of the iodoalbumin employed reflects the biological behavior of endogenous albumin, and this question would appear to be unsettled.

It is clear that little could be said about the distribution effect on observed degradation if a nonhomogeneous labeled material were used. The approach employed here of using the donor as a "biological filter" to screen out more rapidly degraded components would appear to be the method of choice at present to obtain material biologically homogeneous. The general inapplicability of this approach is obvious, however.

The use of a general kinetic formulation has brought to light in a formal way several aspects of iodoalbumin metabolism which apparently have not previously appeared in the literature: the quantitation of transcapillary transport rates of an open system, the probability of a very slowly exchanging or nonexchanging body compartment, the favoring of the rapidly exchanging extravascular compartment as the principal site of iodoalbumin degradation, and kinetic evidence suggesting the physically realizable three-compartment series model to be incompatible with the experimental data.

Little can be said regarding the significance of the postulated Compartment 6. There is experimental evidence that many of the plasma proteins do exist intracellularly in a variety of tissues where they maintain their identity at least to the extent of reacting with specific antibody (20). To our knowledge it has not been demonstrated that these intracellular proteins exchange with circulating plasma proteins. In addition it is known that antigens after injection may enter tissue cells where they may remain, apparently unchanged, for long periods of time. Since heavily iodinated albumin differs antigenically from native albumin (21) it is possible that the labeled protein entering Compartment 6 represents a heavily iodinated component in the injected material which is being treated by the body as an antigen.

The model employed here undoubtedly represents an over-simplification of the biological system. Compartments 2 and 3 probably each represent a composite of tissues having mean turnover rates of the approximate magnitude computed in the analysis. Although the actual biological system could undoubtedly be better represented by a more complex model, it is evident from the data that the system cannot be adequately described by a model of lesser complexity.

SUMMARY

Tracer experiments employing I^{131} labeled iodoalbumin in myxedematous and euthyroid patients indicated a kinetic system containing at least three exchanging compartments and three ancillary compartments (including urinary excretion). Additional studies indicated that the principal excretion forms were urinary iodide and diiodotyrosine. To insure a biologically homogeneous labeled material, one patient was studied in whom the administered labeled albumin was obtained from a donor patient after an eight-day period.

The analysis of the data from the latter tracer study is presented in some detail. The general

solution of a three-compartment open system is given as well as modifications necessary for the solution of a six-compartment open system of the type encountered in the tracer studies. A mapping procedure preserving the invariants of the data was carried out and its value in delineating mathematically compatible and physically realizable models as well as the limits of computed turnover rates for nonunique solutions is illustrated. The numerical effect of variation of the site of entry of newly synthesized carrier into the system on various parameters of the solution is demonstrated.

The solution is further developed in such a way as to permit quantitation of inequalities of final specific activities in the compartments of an open system. Equations for the determination of errors in "intercept," "dilution" and "slope" methods are presented.

Computed values for asymptotic urinary excretion of label suggested the presence of a body compartment having a very slow turnover rate. The parallel model was shown to be one of an infinite number of mathematically compatible and physically realizable models, while the series model was excluded. The analysis suggested that Compartment 2, the rapidly exchanging extravascular compartment, is the principal site of iodoalbumin degradation, irrespective of the model chosen.

REFERENCES

1. Berman, M., and Schoenfeld, R. Invariants in experimental data on linear kinetics and the formulation of models. J. appl. Physics 1956, **27**, 1361.
2. Lewallen, C. G., Rall, J. E., and Berman, M. Studies of iodoalbumin metabolism. II. The effects of thyroid hormone. J. clin. Invest. 1959, **38**, 88.
3. Siri, W. E. Isotopic Tracers and Nuclear Radiations. New York, McGraw-Hill Book Company, Inc., 1949, ch. XV.
4. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 4th ed. Ames, Iowa, The Collegiate Press, 1946, ch. VI.
5. Sterling, K. The turnover rate of serum albumin in man as measured by I^{131}-tagged albumin. J. clin. Invest. 1951, **30**, 1228.
6. Wasserman, K., and Mayerson, H. S. Exchange of albumin between plasma and lymph. Amer. J. Physiol. 1951, **165**, 15.
7. Berson, A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with I^{131}-labeled human serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746.
8. Myant, N. B. Observations on the metabolism of human gamma globulin labeled by radioactive iodine. Clin. Sci. 1952, **11**, 191.
9. Berson, S. A., and Yalow, R. S. The distribution of I^{131} labeled human serum albumin introduced into ascitic fluid: Analysis of the kinetics of a three compartment catenary transfer system in man and speculation on possible sites of degradation. J. clin. Invest. 1954, **33**, 377.
10. Albert, A., and Keating, F. R., Jr. Metabolic studies with I^{131} labeled thyroid compounds: Distribution and excretion of radiodiiodotyrosine in human beings. J. clin. Endocr. 1951, **11**, 996.
11. Skanse, B. Radioactive iodine in the diagnosis of thyroid disease. Acta med. scand. 1949, Suppl. **235**, 1.
12. Riggs, D. S. Quantitative aspects of iodine metabolism in man. Pharmacol. Rev. 1952, **4**, 284.
13. Keating, F. R., Jr., Power, M. H., Berkson, J., and Haines, S. F. The urinary excretion of radioiodine in various thyroid states. J. clin. Invest. 1947, **26**, 1138.
14. Gitlin, D., Janeway, C. A., and Farr, L. E. Studies on the metabolism of plasma proteins in the nephrotic syndrome. I. Albumin, γ-globulin and iron-binding globulin. J. clin. Invest. 1956, **35**, 44.
15. Luck, J. M. On the "turnover" of plasma proteins. Amer. J. Med. 1956, **20**, 317.
16. Armstrong, S. H., Jr., McLeod, K., Wolter, J., and Kukral, J. The persistence in the blood of the radioactive label of albumin, gamma globulins, globulins of intermediate mobility studied with S^{35} and paper electrophoresis: Methods and preliminary results. J. Lab. clin. Med. 1954, **43**, 918.
17. Margen, S., and Tarver, H. Comparative studies on the turnover of serum albumin in normal human subjects. J. clin. Invest. 1956, **35**, 1161.
18. McFarlane, A. S. Labelling of plasma proteins with radioactive iodine. Biochem. J. 1956, **62**, 135.
19. Yalow, R. S., and Berson, S. A. Chemical and biological alterations induced by irradiation of I^{131} labeled human serum albumin. J. clin. Invest. 1957, **36**, 44.
20. Gitlin, D., Landing, B. H., and Whipple, A. The localization of homologous plasma proteins in the tissues of young human beings as demonstrated with fluorescent antibodies. J. exp. Med. 1953, **97**, 163.
21. Friedberg, W., Walter, H., and Haurowitz, F. The fate in rats of internally and externally labelled heterologous proteins. J. Immunol. 1955, **75**, 366.

STUDIES OF IODOALBUMIN METABOLISM. II. THE EFFECTS OF THYROID HORMONE *

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The profound effects of thyroid hormone on a variety of metabolic systems have been well documented. In this report evidence that thyroid hormone alters the distribution kinetics and degradation of iodoalbumin in man is presented. The results of these studies have been previously presented in a preliminary report, prior to refinement of the mathematical analysis employed (1).

METHODS

General. The I^{131} labeled human serum albumin used in these studies was the commercial preparation of Abbott Laboratories obtained between May, 1953, and May, 1954. Prior to use, the material was suspended in redissolved lyophilized plasma and then dialyzed with a cellulose membrane to equilibrium three times against 40 times its volume of a 1:25 dilution of plasma in saline. Without added carrier protein, considerable loss of label on glassware ensued and the preparation was unstable on prolonged dialysis. Quantitation of activity in the dialysate indicated the presence in the preparation of 0.3 to 2.4 per cent dialyzable I^{131} prior to dialysis.

The dialyzed iodoalbumin solution was injected into the rubber tubing of a previously inserted intravenous Murphy drip of normal saline in approximately 20 seconds from a 10 ml. syringe gravimetrically calibrated to deliver with an error not exceeding 0.1 per cent. The injection was immediately followed by a 30 second rinse from the Murphy drip. The intravenous tubing was then clamped, the needle removed, and both thoroughly rinsed into a volumetric flask. Less than 0.1 per cent of the syringe dose remained in the tubing in all experiments.

Heparinized venous blood samples were taken at approximately the following times after injection: 5, 10, 20 and 40 minutes; 1, 2, 4, 6, 8, 12, 18 and 24 hours. In the earlier studies of Experiment I, after 24 hours, blood samples were taken every other day. In subsequent studies, samples were taken daily. In Experiment I samples were collected for 14 days; in Experiment II, for 36 days.

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The heparinized plasma was separated promptly by centrifugation and 1 ml. or 3 ml. samples counted in 4 ml. vials in series with a standard of the same volume consisting of a suitable dilution of the injection suspension in plasma. Volumetric glassware was of precision grade. The counter used was a thallium activated sodium iodide scintillation crystal of the well type. Appropriate corrections were made for coincidence loss. Radioactivity was expressed as fraction of the injected dose.

Total urine collections were made at 24 hour intervals throughout the studies in Experiments I and II, in glass jars containing a few pellets of sodium hydroxide, 5 ml. of toluene, and 4 Gm. of sodium iodide. Counting of aliquots and expression of radioactivity were the same as for plasma. At varying intervals, plasma samples were taken for determination of albumin by sodium sulfate fractionation (2) and gasometric determination of albumin nitrogen after Kjeldahl digestion (3).

Records were kept of the total volumes of blood withdrawn for any reason during the experiments and hematocrits were determined not less frequently than once a week. It was thus possible to compute the fraction of the injected dose removed in blood sampling. In Experiments I and II this amounted to less than 0.01 of the dose and was disregarded in subsequent calculations.

Selection of patients and description of experiments. Two experiments carried out on six patients comprise the basis of this report. Some aspects of these experiments have been discussed in another report (4).

All the patients were under observation for treatment of histologically proven thyroid carcinoma. All had previously had either surgical removal or I^{131} radiation-destruction of their thyroids or both as part of their therapeutic regimen. The myxedematous state was induced by prolonged deprivation of thyroid hormone with or without addition of antithyroid drugs in an attempt to induce therapeutically significant uptake of I^{131} in known or suspected cancerous deposits.

Experiment I. The pertinent clinical data on the four patients (R. B., J. G., F. D. and O. H.) studied in this experiment, in both the euthyroid and myxedematous state, are summarized in Table I. Two patients (R. B. and J. G.) displayed no evidence of local recurrence or distant metastases. The pulmonary metastases of F. D. were not extensive roentgenologically and had been stationary on serial chest films for four years. O. H. had laryngoscopically demonstrable residual tumor in the larynx and subglottic area necessitating a permanent tracheostomy.

TABLE I

General clinical status of patients studied in Experiments I and II

Patient	R. B.	J. G.	F. D.	O. H.	C. C.	E. L.
Age (years), sex	19, F	22, F	18, F	74, M	59, M	60, F
Thyroid carcinoma type	Mixed-follicular, alveolar and papillary	Mixed-papillary, follicular, alveolar and solid	Mixed-papillary and alveolar	Solid	Follicular	Alveolar and follicular
Apparent clinical extent of carcinoma: Local recurrence or metastases	None	None	None	Invasion of trachea and larynx	None	Extensive cervical lymph node involvement
Distant metastases	None	None	Bilateral, basilar pulmonary lesions	None	Small osteolytic metastasis, right clavicle	Extensive sternal and pulmonary lesions
Method of thyroid ablation	Surgical total thyroidectomy followed by 100 mc. I^{131}	Surgical left thyroid lobectomy and partial right lobectomy followed by 78 mc. I^{131}	Surgical right thyroid lobectomy followed by 75 mc. I^{131}	Surgical right thyroid lobectomy followed by 67 mc. I^{131}	78 mc. I^{131}	75 mc. I^{131}
Additional disease	Hypoparathyroidism. Mental retardation	None	None	Generalized arteriosclerosis. Prostatic hypertrophy	Quiescent duodenal ulcer. Arteriosclerotic heart disease, functional capacity Class I	Arteriosclerotic heart disease, functional capacity Class II

At the time of the first iodoalbumin study, all four patients had gross clinical evidences of myxedema. Throughout the study all were fully ambulatory and in good general physical condition, except for the manifestations of myxedema.

In three of the patients (R. B., J. G. and F. D.), addition of antithyroid drugs was not necessary for the induction of myxedema. This alone is fairly good presumptive evidence of the virtual absence of functioning thyroidal tissue. In Patient O. H. it was necessary to add an antithyroid drug (thiouracil) to induce myxedema. This patient, the only one of the four, received therapeutic I^{131} between the first and second iodoalbumin tracer studies

When the presence of myxedema had been established on clinical and laboratory grounds, the first iodoalbumin study was carried out. Later, following return to an apparently euthyroid state and at a time when the body weight was steady, the second tracer study was performed. Data relevant to the metabolic status of the four patients at the time of the studies is given in Table II.

The rate of urinary excretion of I^{131} reflects the rate of degradation of iodoalbumin only in the absence of significant nonrenal loss of I^{131}, and in the absence of entry of the liberated I^{131} into thyroidal iodine metabolism with consequent delay in excretion. For this reason it is desirable to block the uptake of iodide by the thyroidal system during an iodoprotein tracer study. Patients R. B. and O. H. received Tapazole® and thiouracil, respectively, during both studies. J. G. received Tapazole® during the first study. A NaI^{131} tracer performed shortly after the first albumin study and after withdrawal of Tapazole® showed excretion in the urine of 100 per cent of the label in four days. Accordingly no blocking agent was considered necessary during the second study and none was given. Patient F. D., by oversight, received no blocking agent during the first study. However, a NaI^{131} tracer performed six weeks after the first study showed a urinary excretion of label of 0.96 per cent of the dose in four days. Two weeks prior to the second albumin study, this patient underwent cholecystography, a procedure known to inhibit thyroidal iodide uptake for several months. Hence, during both studies, all four patients were either receiving thyroid blocking agents or demonstrated their inability to retain I^{131} presented as iodide ion in amounts exceeding 0.04 of the dose.

In Experiment I the probable error of counting of the least active plasma or urine samples was less than 1.1 per cent. The amount of tracer employed ranged from 80 to 125 μc.

Experiment II. This experiment was performed to study the effects of thyroid hormone on the behavior of the same batch of iodoalbumin in the same patient under different metabolic conditions. Three patients (R. B., C. C. and E. L.) were studied for a period of 36 days. The pertinent clinical background data are given in Table I. All three patients received the same preparation of iodoalbumin in a dosage of 660 μc. In the cases of R. B. and C. C., the metabolic status was changed on the fifteenth day by withdrawal and addition, respectively, of thyroid hormone, dividing the study into Part 1 (Days 0 to 15) and Part 2 (Days 16 to 36). Urine and plasma samples were collected and assayed as described above.

R. B. had been deprived of thyroid hormone for one month prior to the experiment and was showing distinct clinical evidence of hypothyroidism. For a four day period beginning three days prior to injection of labeled albumin,

she was rapidly converted to a slightly hyperthyroid state by administration of L-triiodothyronine in a daily dose of 525 μg. For the remainder of Part 1 of the experiment, the mean daily dose was 348 μg. On Day 15 the hormone was withdrawn. Within a week after its withdrawal, rather definite clinical evidence of hypothyroidism appeared. Data relevant to her changing metabolic status are shown in the lower part of Figure 4. An electrocardiogram four days prior to injection of labeled albumin showed low voltage QRS complexes and low to flat T waves, characteristic of hypothyroidism. By Day 2, the tracing was normal except for sinus tachycardia. On Day 28 low voltage QRS complexes and flat T waves had reappeared. In summary, the clinical picture and laboratory findings were all consistent with mild hyperthyroidism in Part 1, and rapid development of hypothyroidism in Part 2.

Throughout the experiment R. B. received Tapazole®, 40 mg. daily, and saturated solution of potassium iodide, 15 minims daily. After completion of the iodoalbumin study, a NaI^{131} tracer with the patient on the same medication showed a urinary excretion of 0.955 of the label in six days.

Patient C. C. had received no thyroid medication for eight months and had been myxedematous for several months before the experiment. Part 1 of the study was carried out in the myxedematous state. On Day 15, he was started on L-triiodothyronine, 210 μg. daily. After five days the dosage was reduced so that the mean daily dose for Part 2 was 111 μg. daily. During the last eight days of the experiment desiccated thyroid, 90 mg. daily, was also given along with appropriate reduction of triiodothyronine so as to maintain a clinically euthyroid state. Data relative to the patient's changing metabolic status are shown in Figure 5. In addition, the average of 10 determinations of the basal metabolic rate (BMR) in Part 1 was −36 per cent; in Part 2, −9 per cent.

Thiouracil, started on Day 1 of the experiment, was discontinued on Day 3 because of drug rash. However, a NaI^{131} tracer given just prior to the albumin study showed a urinary excretion of label of 0.945 in three days.

Patient E. L. served as a control and apparently remained in a steady metabolic state throughout the 36 days of the study. Though having received several large therapeutic doses of I^{131} in the previous few years, she was

TABLE II

Data relevant to metabolic status of patients studied in Experiment I

Patient		R. B.	J. G.	F. D.	O. H.
Period of thyroid hormone deficiency prior to first study (*months*)		5	5	5	6
Body weight (*Kg.*)	(1)*	55.1	65.3	67.6	71.8
	(2)*	48.5	60.8	62.0	69.6
Serum cholesterol (*mg. %*)	(1)	391	422	390	499
	(2)	148	254	140	313
Protein bound iodine (μg. %)	(1)	1.6	1.7	1.0	0.8
	(2)	8.2	5.7	4.7	7.3
Basal metabolic rate (%)	(1)	−45	−41	−44	−36
	(2)	−12	−20	−28	Unsatisfactory
Desiccated thyroid dosage and duration prior to second study		240 mg. daily 30 days	120 mg. daily 23 days	120 mg. daily 30 days	120 mg. daily 90 days
Thyroid blocking agent	(1)	Tapazole® 40 mg. daily	Tapazole® 40 mg. daily	None	Thiouracil 1.5 Gm. daily
	(2)	Tapazole® 40 mg. daily	None	Cholecystography	Thiouracil 1.5 Gm. daily
4 day I^{131} iodide† excretion (fraction of dose)			1.00	0.96	
Mean serum albumin (*Gm. %*)	(1)	5.03	5.20	4.79	4.32
	(2)	4.28	4.94	4.29	3.99
Mean serum globulin (*Gm. %*)	(1)	2.94	2.73	2.50	2.90
	(2)	2.89	2.57	2.60	2.77

* (1) refers to indices measured in the myxedematous state at the time of the first tracer study, (2) to measurements made at the time of the second study in the euthyroid state.

† These NaI^{131} tracers were performed in the absence of thyroid hormone or thyroid blocking agents.

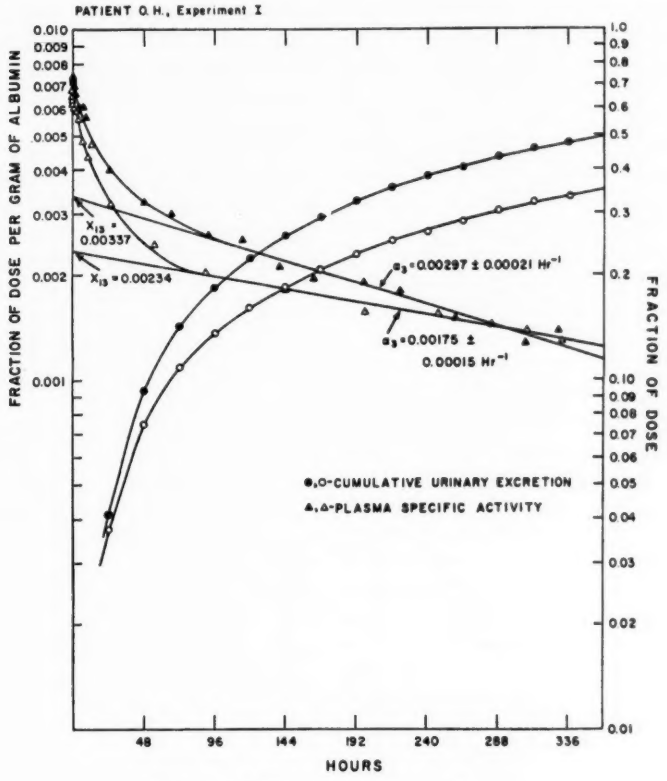
PATIENT O. H., Experiment I
FRACTION OF DOSE PER GRAM OF ALBUMIN
FRACTION OF DOSE
HOURS
X_{13} = 0.00337
X_{13} = 0.00234
α_3 = 0.00297 ± 0.00021 Hr^{-1}
α_3 = 0.00175 ± 0.00015 Hr^{-1}
●,○-CUMULATIVE URINARY EXCRETION
▲,△-PLASMA SPECIFIC ACTIVITY

FIG. 1. I^{131}-IODOALBUMIN TRACER STUDIES

Open points indicate myxedema study; shaded points, euthyroid study.

known to have residual functioning thyroid cancer in the neck, lungs and sternum. Despite the fact that she had never received exogenous thyroid hormone, she had never developed myxedema but had shown mild hypothyroidism for the previous few years. Presumably, her tumor was producing significant but less than normal amounts of thyroid hormone. Neither antithyroid drugs nor iodide was given during the study because of the possibility of altering her metabolic status or interfering with contemplated I^{131} therapy. During the study she appeared to be in a mildly hypothyroid steady state. Her weight varied from 70.4 to 71.3 Kg., her serum cholesterol from 171 to 215 mg. per cent, and her average BMR was −5 per cent. A NaI^{131} tracer performed shortly after completion of the iodoalbumin study showed only 0.60 urinary excretion of label in four days. Hence, little significance could be attached to her urinary excretion data from the iodoalbumin study.

In Experiment II the probable error of counting for the least active plasma and urine samples did not exceed 1.4 and 3.8 per cent, respectively.

In addition to the iodoalbumin studies, all patients were subjected to ancillary NaI^{131} tracer studies in either the euthyroid or myxedematous state, and the rate constant of renal iodide excretion, Kie, was computed by the approach described by Keating, Power, Berkson and Haines (5). In those instances in which Kie was determined in only one metabolic state, Kie for the other was computed from the relationship obtained from the data of Keating, Haines, Power and Williams:

$$\frac{\text{Kie (myxedema)}}{\text{Kie (euthyroid)}} \cong 0.76 \text{ (6)}.$$

RESULTS AND ANALYSIS OF DATA

The notation and terminology employed in the discussion to follow are the same as that defined elsewhere (4).

The plasma and urinary excretion data of Patient O. H., typical of the studies of Experiment I, are shown in Figure 1. It is noteworthy that in myxedema the values of X_{13} and α_3 are considerably smaller than the respective values in the euthyroid state, and that urinary excretion

TABLE III

Data obtained by graphic analysis of I^{131}-albumin and NaI^{131} tracer studies

Patient	O. H.	J. G.	J. G.	F. D.	F. D.	R. B.	R. B.	R. B.	R. B.	C. C.
Metabolic status	Euthyroid	Myxedema	Euthyroid	Myxedema	Euthyroid	Myxedema	Hyperthyroid	Euthyroid	Transitional	Myxedema	Myxedema
Experiment	I	I	I	I	I	I	III	I	II, Part I	I	II, Part I
A_{11}	0.222	0.098	0.140	0.271	0.152	0.236	0.146	0.148	0.103	0.229	0.392
A_{12}	0.311	0.542	0.443	0.407	0.374	0.415	0.326	0.313	0.387	0.342	0.356
A_{13}	0.467	0.360	0.417	0.322	0.474	0.349	0.528	0.539	0.510	0.429	0.252
α_1	0.173	0.502	0.213	0.321	0.183	0.204	0.180	0.231	0.312	0.315	0.221
α_2	0.0385	0.0513	0.0415	0.0488	0.0358	0.0408	0.0301	0.0420	0.0436	0.0426	0.0312
α_3	0.00297	0.00175	0.00232	0.00166	0.00293	0.00181	0.00339	0.00322	0.00311	0.00186	0.00123
A_{55}	0.765	0.719	0.990	0.743	0.820	0.725	0.861	0.820	0.886	0.654	0.893
A_{53}	−0.778	−0.691	−0.942	−0.695	−0.773	−0.711	−0.866	−0.791	−0.830	−0.638	−0.825
ΣX_{1i}	0.00722	0.00651	0.00720	0.00738	0.00810	0.00868	0.00970	0.00811	0.00750	0.00821	0.00783
λ_{54}	0.0269	0.0202	0.0587	0.0440	0.0558	0.0418	0.0739	0.0739	0.0739	0.0375	0.0329
$A_{21} + A_{31}$							−0.176				
$A_{22} + A_{32}$							−0.255				
$A_{23} + A_{33}$							0.431				

of label is appreciably faster in euthyroidism. (The first five points of the myxedema plasma curve have been shifted slightly to the left by the artist to avoid overlapping the euthyroid plasma curve.)

The plasma and urine data of Experiment I and that of Patients C. C. and R. B., Experiment II, Part 1, were subjected to graphic analysis as described elsewhere (4). The data of E. L., Experiment II, were not subjected to compartmental analysis because of the presence of unblocked, functioning metastases which presumably vitiated her excretion data. As before, the values of α_3 and X_{13} were obtained by the method of least squares. The constants obtained from the experimental data are shown in Table III. Due to the fact that urine collections were not made more frequently than every 24 hours, graphical differentiation of the early part of the excretion curves could not be carried out and neither could the exponential components of the early portions of the excretion curves be resolved. Only the A_{55} and A_{53} components from the terminal portions of the curves could be reliably determined from the data.

The formulation of an open system of three exchanging compartments and three nonexchanging compartments and its mathematical solution have been described previously. When the most reliable and complete set of iodoalbumin tracer data at our disposal (R. B., Experiment III) were subjected to this analysis, the best obtainable fit of the data occurred with $\lambda_{01} = 0$. The data of R. B., Experiment III, were shown to contain seven of the nine experimentally measured constants required for a complete solution of the general system of three exchanging compartments. The solution was therefore not unique. Four "simplest" models were shown to exist that are compatible with the data, physically realizable, and equally probable to be the "right model" on the basis of the tracer data only (4). One of these, the parallel model, was considered most likely on the basis of physiological considerations and is the model arbitrarily chosen for this presentation.

It should be noted that the data shown in Table III (excepting R. B., Experiment III) contain as such only five of the nine independent constants of a general system of three exchanging

compartments: three α's and two A's from $q_1(t)$. The additional measured values of A_{55}, A_{53} and λ_{54} permit evaluation of a sixth independent constant $(A_{23} + A_{33})$ from the relation

$$(A_{23} + A_{33}) = -A_{13} + \frac{A_{53}\alpha_3}{A_{55}\lambda_{54}} - \frac{A_{53}}{A_{55}}.$$

This equation follows from the general relation, $\sum_j A_{ji} = 0$, for the multicompartment system previously described and is independent of the configuration of the three exchanging compartments. The solutions for the tracer studies reported here follow the same procedures as outlined in our previous paper (4) with the three constraints that $\lambda_{23} = \lambda_{32} = \lambda_{01} = 0$. Newly synthesized carrier is assumed to enter Compartment 1. The assumptions regarding Compartment 6 are the same as those previously given. The model is shown in Figure 2.

The results of the solution are given in Table IV. In the extreme right column is given, for several of the solution parameters, the mean ratio of their value in myxedema to their value in the euthyroid state. Patient C. C. was not studied in a euthyroid steady state. The results

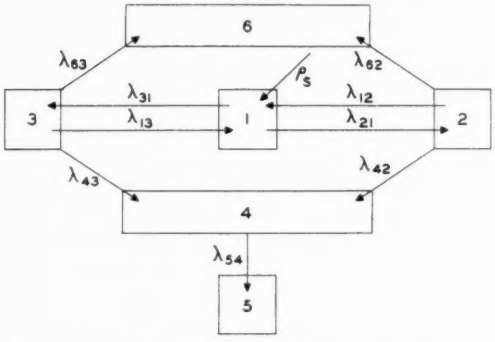

FIG. 2. DIAGRAMMATIC REPRESENTATION OF MODEL USED FOR ANALYSIS OF DATA OF EXPERIMENTS I AND II

The model consists of three exchanging compartments (1, 2 and 3) and three nonexchanging compartments (4, 5 and 6). Compartment 1 is plasma, the sampled compartment. Compartments 2 and 3 are, respectively, rapidly and slowly exchanging extravascular compartments. Compartment 4 represents nonexcreted degradation products. Compartment 5 is collected urine. Compartment 6 is a body compartment treated analytically as a nonexchanging compartment. Newly synthesized carrier is assumed to enter Compartment 1 at the rate ρ_s. The model shown assumes $(\lambda_{61} + \lambda_{41}) \equiv \lambda_{01} = \lambda_{32} = \lambda_{23} = 0$.

of the myxedema study (Experiment II, Part 1) are included because this patient showed the highest value of S_T and the lowest value of λ_{4T} encountered in the studies. The results of the study on R. B., Experiment III, published elsewhere (4), are included here to show the stepwise change seen in the solution parameters under the conditions of varied metabolic status.

It should be noted that in all the studies listed, except that of R. B., Experiment III, the values of λ_{43} and λ_{63} and the corresponding ρ's are of negative sign. This represents a systematic inconsistency between the data and the model used for the analysis. Further study of the inconsistency by the mapping procedure described previously (4) indicated that the inconsistency does not result from choice of the parallel model since a mapping of the data did not show a region of physical realizability. An average increase in the value of $(A_{23} + A_{33})$ of 7 per cent resulted in a physically realizable solution for all the tracer studies, and one way by which a realizable fit to the parallel model could be obtained was to increase the value of $(A_{23} + A_{33})$ by at most 25 per cent. This systematic inconsistency could also result from a systematic error in evaluating λ_{54}, nonvalidity of the assumption that $q_6(t)$ is proportional to $q_4(t) + q_5(t)$, or from the presence in the labeled material of components degraded at different rates (7). In view of the relatively small magnitude of the inconsistency it was considered preferable to present the solution in its inconsistent form rather than to introduce additional assumptions.

When constraints are introduced and inconsistencies are encountered (as here) it is advisable to consider which solution parameters are independent of the constraints. In our previous paper (4) it was pointed out that S_1, λ_{11} and $\lambda_{22} + \lambda_{33}$ are dependent only on time measurements of Compartment 1 and are thereby independent of the choice of model. Granted $\lambda_{01} = 0$ (evidence restricted to data of Experiment III), $\lambda_{21} + \lambda_{31}$ is constant for all models. Granted, in addition, entry of new carrier into Compartment 1, $S_2 + S_3$ and $S_1 + S_2 + S_3$ are constant for all models.

Table IV shows an average increase of S_T in myxedema of 31 per cent. All of this increase can be accounted for by the 70 per cent increase

TABLE IV

Results of the open parallel model solution *

Patient	O. H.	O. H.	J. G.	J. G.	F. D.	F. D.	R. B.	R. B.	R. B.	R. B.	C. C.	Mean ratio†
Metabolic status	Euthyroid	Myxedema	Euthyroid	Myxedema	Euthyroid	Myxedema	Hyper-thyroid	Euthyroid	Transitional myxedema→hyperthyroid	Myxedema	Myxedema	Myxedema/ euthyroid
Experiment	I	I	I	I	I	I	III	I	II, Part 1	I	II, Part 1	
λ_{11}	0.0518	0.0776	0.0492	0.107	0.0426	0.0657	0.0379	0.0491	0.0506	0.0875	0.0981	1.75
λ_{22}	0.139	0.456	0.187	0.242	0.158	0.162	0.156	0.200	0.282	0.248	0.142	1.71
λ_{33}	0.0237	0.0214	0.0210	0.0218	0.0210	0.0190	0.0197	0.0275	0.0260	0.0241	0.0130	
λ_{21}	0.0382	0.0516	0.0300	0.0777	0.0301	0.0411	0.0275	0.0363	0.0362	0.0678	0.0745	1.79
λ_{31}	0.0136	0.0261	0.0192	0.0297	0.0125	0.0246	0.0104	0.0128	0.0144	0.0197	0.0235	1.74
λ_{13}	0.0292	0.0249	0.0231	0.0249	0.0253	0.0197	0.0192	0.0326	0.0312	0.0272	0.0146	
λ_{12}	0.106	0.377	0.142	0.214	0.114	0.139	0.124	0.155	0.214	0.224	0.128	1.94
λ_{42}	0.0255	0.0568	0.0441	0.0210	0.0359	0.0166	0.0278	0.0366	0.0600	0.0159	0.0129	
λ_{62}	0.00782	0.0222	0.000455	0.00728	0.00788	0.00630	0.00448	0.00803	0.00775	0.00843	0.00155	
λ_{43}	−0.00419	−0.00255	−0.00207	−0.00226	−0.00353	−0.000508	0.000451	−0.00424	−0.00464	−0.00199	−0.00146	
λ_{63}	−0.00128	−0.000997	−0.0000213	−0.000784	−0.000775	−0.000193	0.0000726	−0.000931	−0.000600	−0.00105	−0.000176	
λ_{4T}	0.00249	0.00142	0.00250	0.00136	0.00266	0.00139	0.00302	0.00284	0.00304	0.00131	0.00123	0.53
λ_{6T}	0.00325	0.00198	0.00252	0.00184	0.00324	0.00192	0.00351	0.00346	0.00343	0.00200	0.00138	0.63
S_1	138.5	153.6	138.9	135.5	123.5	115.2	103.1	123.3	133.3	121.8	127.7	1.00
S_2	38.0	17.4	22.3	43.5	23.5	29.2	18.2	22.4	17.1	33.3	66.8	
S_3	79.5	187.0	127.2	184.5	73.3	149.4	54.3	57.4	74.0	99.5	231.4	1.90
S_T	256.0	358.0	288.4	363.5	220.3	293.8	175.6	203.1	224.5	254.7	426.0	1.31
$S_2 + S_3$	117.5	204.4	149.5	228.0	96.9	178.6	72.5	79.8	91.1	132.9	298.3	1.70
ρ_0	0.832	0.710	0.727	0.667	0.714	0.564	0.616	0.703	0.770	0.509	0.586	0.82
ρ_{11}	5.29	7.92	4.16	10.5	3.72	4.73	2.83	4.48	4.82	8.26	9.52	1.79
ρ_{21}	1.89	4.00	2.67	4.02	1.54	2.84	1.07	1.57	1.92	2.40	3.01	1.75
ρ_{12}	4.02	6.55	3.17	9.30	2.69	4.06	2.25	3.48	3.67	7.45	8.55	2.06
ρ_{13}	2.32	4.66	2.94	4.59	1.86	2.94	1.04	1.87	2.31	2.70	3.39	1.65
ρ_{42}	0.969	0.986	0.983	0.913	0.844	0.485	0.506	0.819	1.03	0.531	0.861	
ρ_{62}	0.297	0.386	0.0101	0.316	0.185	0.184	0.0815	0.180	0.133	0.281	0.103	
ρ_{43}	−0.333	−0.476	−0.263	−0.418	−0.259	−0.0759	0.0245	−0.243	−0.344	−0.198	−0.339	
ρ_{63}	−0.102	−0.187	−0.00271	−0.145	−0.0569	−0.0288	0.00394	−0.0534	−0.0444	−0.105	−0.0406	
Plasma volume (liters)	3.44	3.56	2.81	2.61	2.88	2.41		2.88		2.42		0.91

* The number of digits appearing in the solution parameters represents an arbitrary rounding off and is not intended to reflect experimental precision.

† Computed only from Experiment I. Mean ratios are shown only for those parameters showing marked or consistent change or change of particular interest.

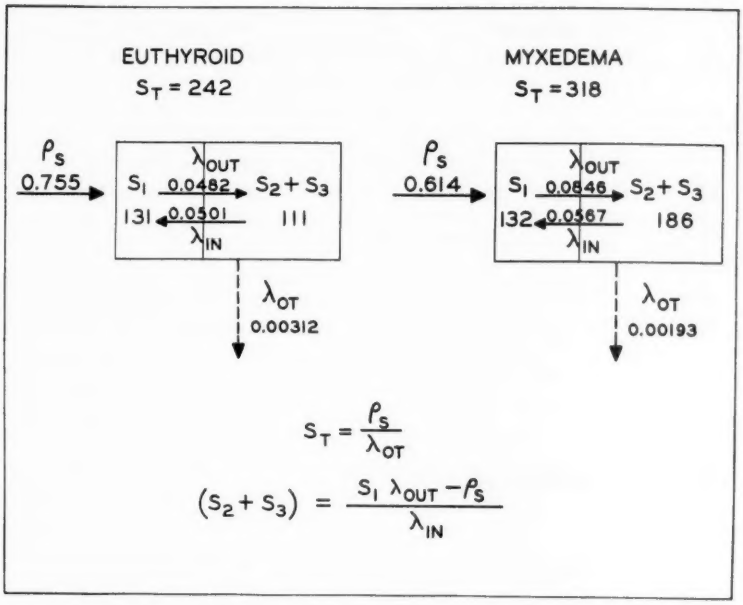

FIG. 3. SIMPLIFIED DIAGRAMMATIC REPRESENTATION OF ALBUMIN METABOLISM KINETICS IN EUTHYROIDISM AND MYXEDEMA

in extravascular carrier, $S_2 + S_3$, since there is no appreciable change in plasma albumin, S_1. Furthermore, the increase in $S_2 + S_3$ is almost entirely accounted for by the 90 per cent increase in S_3, the carrier of the slowly exchanging extravascular compartment.

Some insight into the development of this situation may be gained from the following considerations. In Figure 3, for purposes of clarity, the model has been simplified by combining Compartments 2 and 3 into a single extravascular compartment. In the figure

$$\lambda_{out} \equiv \lambda_{21} + \lambda_{31},$$

$$\lambda_{in} \equiv \frac{\rho_{12} + \rho_{13}}{S_2 + S_3},$$

the latter to give an average value for the fractional rate of transport of albumin from the extravascular compartments to plasma. The average state of affairs in euthyroidism and myxedema is depicted. The solution values shown are the mean values of the parameters in the two metabolic states taken from Table IV with the exception of λ_{in} and ρ_s which were altered slightly (less than 4 per cent) to "balance" the models shown.

In any given steady state where the rate of carrier synthesis is equal to the rate of carrier loss, the following relationship must hold:

$$\rho_s = S_T \lambda_{0T}, \quad S_T = \frac{\rho_s}{\lambda_{0T}}.$$

Consideration of this relationship and Figure 3 shows that, from a descriptive standpoint at least, the increase in S_T in myxedema is associated with a marked reduction in λ_{0T} and cannot be attributed to a change in the rate of synthesis, since a considerable increase in the carrier of the system occurs despite a reduction in the rate of synthesis. It is of considerable interest that the increase in total carrier, explainable in terms of a reduction in the fractional rate of loss of carrier from the system as a whole, is confined to the extravascular compartments. From Figure 3 it is apparent:

$$(S_2 + S_3)\lambda_{in} + \rho_s = S_1 \lambda_{out}$$

$$S_2 + S_3 = \frac{S_1 \lambda_{out} - \rho_s}{\lambda_{in}}.$$

Consideration of this relationship and Figure 3 reveals that the confinement of the increase in carrier in myxedema to extravascular sites is

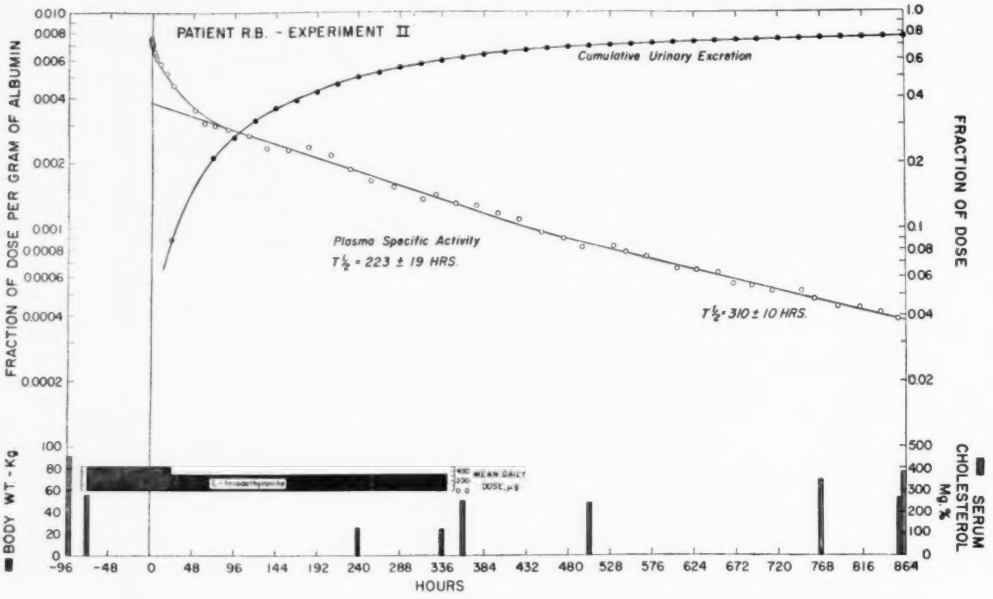
PATIENT R.B. - EXPERIMENT II
Cumulative Urinary Excretion
Plasma Specific Activity
T½ = 223 ± 19 HRS.
T½ = 310 ± 10 HRS.
FRACTION OF DOSE PER GRAM OF ALBUMIN
FRACTION OF DOSE
BODY WT. - Kg
SERUM CHOLESTEROL Mg. %
MEAN DAILY DOSE, μg
HOURS

FIG. 4. THE EFFECTS OF THE WITHDRAWAL OF THYROID HORMONE ON PLASMA SPECIFIC ACTIVITY AND URINARY EXCRETION OF LABEL

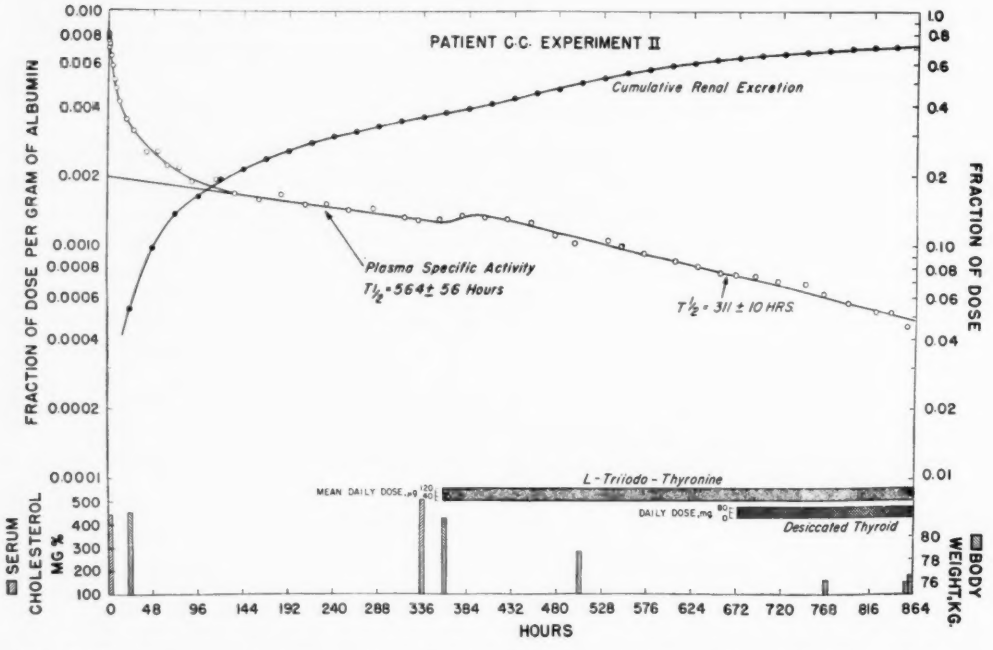

FIG. 5. THE EFFECTS OF ADMINISTRATION OF THYROID HORMONE TO A MYXEDEMATOUS SUBJECT ON PLASMA SPECIFIC ACTIVITY AND URINARY EXCRETION OF LABEL

primarily a reflection of the marked increase in λ_{out}, since the change in S_1 is insignificant. The change in ρ_s contributes little to the right side of the equation, and the change in λ_{in} is in the wrong direction to effect an increase in $S_2 + S_3$. Table IV shows that a marked increase in the outward transcapillary transport rate of albumin was a consistent finding in these studies.

Altogether, Patient R. B. was studied in four metabolic states. The results of all four studies show a gradation of change in the solution parameters. The solution described above is applicable only to the steady state ($\rho_s = S_T\lambda_{0T}$). The study designated "transitional" thyroid status was performed in a presumably unsteady state at a time when the patient was being rapidly converted from a myxedematous to a hyperthyroid status with triiodothyronine. Strictly speaking, this study is not amenable to the analysis outlined. However, it was considered likely that the hormone was exerting its effects primarily on the rates in the system and that changes in compartment sizes during the time of the study would probably be of insufficient magnitude to alter seriously the validity of the analysis. Attention is called to the orderly change in many of the solution parameters proceeding in sequence through the metabolic states designated as hyperthyroid, euthyroid, transitional and myxedematous.

In the studies of Experiment I, each patient served as his own control as far as metabolic status is concerned. However, in any one patient the euthyroid and myxedema studies were carried out with different batches of iodoalbumin, so that conceivably the results shown in the table might be explainable, at least in part, by differences in the tracer material used in the myxedema and euthyroid studies. In part, Experiment II was performed to study this possibility. The plasma specific activity and urinary excretion curves for the three patients are shown in Figures 4, 5 and 6. The patient, E. L., whose metabolic status was unchanged, shows a single final component of plasma specific activity after the "mixing" phase. The decrease in slope of the plasma curve produced by withdrawal of thyroid hormone in R. B. and the increase in slope produced by administration of hormone in C. C. are statistically significant. Compartment analysis of Part 2 of these experiments (after withdrawal and addition of hormone, respectively) is of course not possible, and, as previously shown (4), the final plasma slopes cannot be regarded as more than an approximation to λ_{0T}. It is evident, however, that the changes in slope observed are similar in direction and magnitude to the changes in λ_{0T} noted in the "before and after" type of studies in Experiment I. This would appear to eliminate the possibility that the changes in λ_{0T} noted in Experiment I are a result of some factor other than hormone action. All three patients in this experiment were given

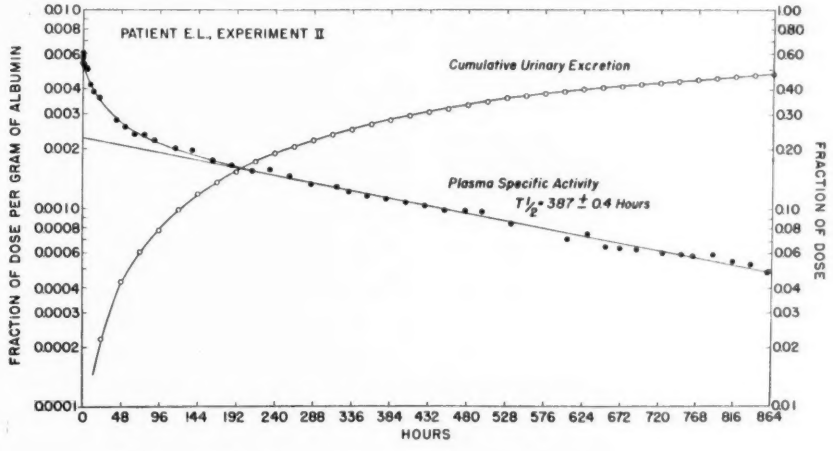

FIG. 6. 36 DAY TRACER STUDY IN A MILDLY HYPOTHYROID PATIENT

The metabolic status underwent no apparent change during the study.

identical aliquots of the same batch of iodoalbumin on the same day.

DISCUSSION

As noted above, the overall increase in carrier albumin in myxedema is associated, at least descriptively, with a reduction in the overall fractional rate of loss of carrier, λ_{0T}, from the system. The analysis indicates that thyroid hormone effects an increase in the absolute rates of synthesis, ρ_s, and of loss, ρ_L. Furthermore, confinement of the increase in carrier albumin in myxedema to extravascular sites would appear to be a result of the increase in outward transcapillary transport rate of albumin, rather than of some factor slowing the return of albumin from extravascular sites to plasma.

It is of some interest to speculate whether the primary effect of the hormone is on the fractional rate of loss of carrier, λ_{0T}, or on the absolute rate of loss, ρ_L. We favor the former rather than the latter possibility. If the former were correct, one would expect hormone administration to produce an increased rate of synthesis, ρ_s', and an increased fractional rate of loss λ_{0T}', and that this would produce a progressive reduction in S_T and automatically reach a new steady state where $\rho_s' = S_T'\lambda_{0T}' = \rho_L'$. This point of view requires no direct coupling of the rates of synthesis and degradation which are, however, equal in any given steady state.

With the latter possibility one would have to postulate from hormone administration an increased rate of synthesis, ρ_s', and of loss, ρ_L', where $\rho_L' > \rho_s'$. This would result in a progressive reduction in S_T but one is then left with the problem of some compensatory mechanism whereby, as the new steady state is approached, either ρ_L' or ρ_s' is again changed such that $\rho_L' = \rho_s'$. Furthermore, with the latter possibility the change in λ_{0T} is a secondary one, and since $\lambda_{0T}' = \frac{\rho_L'}{S_T'}$, it is clear that during the transition phase after administration of hormone, one would not expect to see the full change in λ_{0T} until S_T had reached its final, minimal value. In this connection it is of interest that the "transitional" study on R. B. shows a euthyroid value for λ_{0T} despite the fact that an elevation of S_T still persists. The nonvalidity of the application of the kinetic analysis to this particular study, however, allows little weight to be placed on this evidence. Additional evidence on this point is available from the studies of Gitlin, Janeway and Farr (8) who showed that plasmaphoresis in dogs and chronic bleeding in mice did not increase the fractional rate of catabolism of homologous albumin.

The questionable validity of iodoalbumin as a tracer for native albumin catabolism has been discussed elsewhere (4). In addition, the studies of Steinfeld and co-workers (9) show a more rapid degradation-excretion of the particular type of iodoalbumin employed by us than that from several other preparations tested. In view of this, the absolute values of ρ_s and λ_{0T} shown in Table IV should be regarded as only approximations, probably in error by overestimation. [The validity of the tracer material used in the study of R. B., Experiment III, is less subject to question (4).]

Despite these objections, the controlled manner in which Experiment I was carried out and the results of Experiment II would appear to leave little doubt as to the effect of both desiccated thyroid and L-triiodothyronine on the distribution kinetics and degradation of the iodoalbumin preparation used.

The results of these kinetic studies of serum albumin behavior are more meaningful when considered in conjunction with certain aspects of its metabolism revealed by other approaches. There is excellent experimental evidence that the liver (at least in the rat) is the dominant, if not the sole, site of albumin synthesis (10, 11). In the isolated perfused rat liver albumin labeled in the liver is capable of entering the perfusion system via the hepatic veins (10). Once in the circulating blood, albumin is transported across the capillary wall to the interstitial fluid and body cavity fluids. It is probable that the transport of albumin from the interstitial fluid to plasma is almost exclusively via lymphatics rather than by re-entry of blood capillaries (12). Under normal conditions tracer studies reveal a roughly equal partition of total exchangeable albumin between intravascular and extravascular sites (13, 7). On the basis of biopsy and autopsy studies of patients given I^{131}-albumin Rothschild, Bauman, Yalow and Berson computed that

plasma, interstitial fluid of skin and interstitial fluid of muscle accounted for 40, 18 and 15 per cent, respectively, of total exchangeable albumin (14). Utilizing the fluorescent antibody technique, Gitlin, Landing and Whipple (15) have demonstrated homologous serum albumin intracellularly in a variety of human tissues. In addition, it seems clear that circulating albumin may serve as a general body nitrogen source (16).

Studies similar to those of Experiment I of this paper were carried out by Schwartz on two patients with myxedema and analyzed by slope, intercept and dilution methods (17). Although the data in one patient did not permit satisfactory comparison of plasma slopes before and after treatment, the findings relative to the changes in total exchangeable albumin, extravascular albumin, total turnover rate and rate of synthesis produced by thyroid hormone in myxedema are in substantial agreement with the results reported here.

In experiments conducted by Dixon, Talmage, Maurer and Deichmiller (18) employing I^{131}-gamma globulin, thyroxine administered to rabbits in a dosage sufficient to double their oxygen consumption resulted in a 30 per cent increase in the final plasma slope. Similarly, Rothschild, Bauman, Yalow and Berson (19) administered I^{131}-albumin to previously euthyroid patients receiving large doses of desiccated thyroid. Increases in the fractional rate of degradation, the absolute rate of degradation and the synthetic rate of serum albumin were observed along with a reduction in total exchangeable albumin.

The results of the present study and those of other investigators cited above (17, 19) are in substantial agreement regarding the increase in rate of synthesis, increase in fractional rate of catabolism and reduction of total exchangeable albumin produced by thyroid hormone. These studies, however, shed no light on the mechanism whereby these changes are effected. Steinbock and Tarver have shown that the turnover rate of plasma protein is directly related to the protein content of the diet (20). This aspect of the problem was not controlled in our experiments. It would appear unlikely, however, that the changes observed here were produced by a change in protein intake. In the study of C. C., Experiment II (see Figure 5), an increase in the rate of renal excretion of label was evident as early as 48 hours after administration of triiodothyronine. At this time the patient was experiencing the anorexia, malaise and headache commonly seen in myxedematous patients in the first few days after the start of thyroid hormone.

Whether the observed results are a direct or indirect effect of thyroid hormone is not clear. Although the effects of thyroid hormone on overall nitrogen metabolism are not necessarily relevant to its effect on the metabolism of a single plasma protein, the overall catabolic effect of thyroid hormone in both euthyroid and myxedematous subjects has been well documented (21–23). Studies in rats with N^{15}-glycine by Hoberman and Graff (24) suggest that thyroxine decreases amino acid catabolism and increases the breakdown of body protein to amino acids. The marked increase in transcapillary transport of albumin in the present studies was a somewhat unexpected but consistent finding. The data of Lange are of interest in this connection (25). This investigator injected fluorescein intravenously and showed that the dye appeared in the skin of myxedematous subjects much more rapidly than in normal controls. He concluded that there is an increase in capillary permeability in myxedema.

On the other hand, White and Jones (26), using the pressure plethysmograph, observed in myxedema a reduction in the rate of filtration of fluid across the capillary bed of the arm in response to venous congestion. However, it has been demonstrated by Pappenheimer and Soto-Rivera (27) that in the mammalian circulatory system the permeability of the capillary walls to protein need not necessarily be related to its permeability to fluid.

The anatomical changes in the capillaries of the human ungual fold in myxedema have been studied in the living state by Zondek, Michael and Kaatz (28). The principal changes observed consisted of reduction in the number of visible capillaries, an increase in the intercapillary spaces, a reduction in caliber, a reduction in tortuosity and a loss of the normal discrepancy between the widths of the arterial and venous limbs.

We have no satisfactory explanation for the observed increase in the rate of transcapillary passage of albumin in myxedema. An increase

in capillary pressure and severe anoxia have been shown to produce an increase in the rate of passage of protein across the capillary wall. In studies by Landis, Jonas, Angevine and Erb, a venous congestion pressure above 60 mm. of mercury was required to demonstrate the effect (29). In edema fluid produced by prolonged venous congestion Stead and Warren demonstrated an increase in protein concentration by a congestion pressure of 30 mm. Hg (30). In these instances, the resulting capillary pressure is probably approximately 8 to 14 mm. Hg greater than the congestion pressure (31), such that the increase in capillary pressure above normal is at least 50 per cent. We are not aware of any direct determinations of capillary pressure in myxedema. The mean increase in plasma colloid osmotic pressure in myxedema in the patients of Experiment I computed from their plasma protein concentrations was only 15 per cent (32). Although the mean capillary pressure cannot be computed from the plasma colloid osmotic pressure without assumptions regarding interstitial fluid colloid osmotic pressure and tissue pressure (27), these considerations suggest that the changes in capillary pressure in myxedema, if any, are small, and are probably not the explanation for the marked increase in transcapillary passage of albumin. Although severe anoxia will produce an increase in capillary permeability to both water and plasma protein (33), Stead and Warren (30) found low protein concentrations in the edema fluid from patients with severe reduction in arterial oxygen saturation, from which they concluded that anoxia of a degree sufficient to produce an increased capillary permeability to protein was incompatible with life.

The mean change in plasma volume observed after administration of thyroid hormone (an increase of 10 per cent) is less than the 23 per cent increase reported by Thompson (34).

SUMMARY AND CONCLUSIONS

Four patients with myxedema were subjected to tracer studies with I^{131}-iodoalbumin before and after treatment with thyroid hormone. Two patients were studied by altering their metabolic status during a tracer study. One patient was given tracer studies in four metabolic states. The data were analyzed with reference to an open, multicompartment parallel model.

The analysis indicated that there is in myxedema an increase in total exchangeable albumin which is confined to the extravascular compartments, a reduction in the rate of albumin synthesis and catabolism, a reduction in the fractional rate of albumin catabolism, an increase in the transcapillary transport rate of albumin and a reduction in plasma volume. In patients in whom the metabolic status was altered during tracer experiments the changes in the urinary excretion and plasma curves were consistent with the results of steady state tracer experiments in the euthyroid and myxedematous states.

REFERENCES

1. Lewallen, C. G., Rall, J. E., Berman, M., and Hamel, H. The effects of thyroid hormone on the distribution kinetics and degradation of iodoalbumin (abstract). J. clin. Invest. 1955, **34**, 949.
2. Kingsley, G. R. A rapid method for the separation of serum albumin and globulin. J. biol. Chem. 1940, **133**, 731.
3. Hiller, A., Plazin, J., and Van Slyke, D. D. A study of conditions for Kjeldahl determination of nitrogen in proteins. Description of methods with mercury as catalyst, and titrimetric and gasometric measurements of the ammonia formed. J. biol. Chem. 1948, **176**, 1401.
4. Lewallen, C. G., Berman, M., and Rall, J. E. Studies of iodoalbumin metabolism. I. A mathematical approach to the kinetics. J. clin. Invest. 1959, **38**, 66.
5. Keating, F. R., Jr., Power, M. H., Berkson, J., and Haines, S. F. The urinary excretion of radioiodine in various thyroid states. J. clin. Invest. 1947, **26**, 1138.
6. Keating, F. R., Jr., Haines, S. F., Power, M. H., and Williams, M. D. The radioiodine-accumulating function of the human thyroid gland as a diagnostic test in clinical medicine. J. clin. Endocr. 1950, **10**, 1425.
7. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with I^{131} labeled human serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746.
8. Gitlin, D., Janeway, C. A., and Farr, L. E. Studies on the metabolism of plasma proteins in the nephrotic syndrome. I. Albumin, γ-globulin and iron-binding globulin. J. clin. Invest. 1956, **35**, 44.
9. Steinfeld, J. L., Paton, R. R., Flick, A. L., Milch, R. A., Beach, F. E., and Tabern, D. L. Distribution and degradation of human serum albumin labeled with I^{131} by different techniques. Ann. N. Y. Acad. Sci. 1957, **70**, 109.

10. Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F. The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-ε-C^{14}. J. exp. Med. 1951, **94**, 431.
11. Miller, L. L., Bly, C. G., and Bale, W. F. Plasma and tissue proteins produced by non-hepatic rat organs as studied with lysine-ε-C^{14}. Gamma globulins the chief plasma protein fraction produced by non-hepatic tissues. J. exp. Med. 1954, **99**, 133.
12. Yoffey, J. M., and Courtice, F. C. Lymphatics, Lymph, and Lymphoid Tissue. Cambridge, Mass., Harvard University Press, 1956, ch. 2.
13. Sterling, K. The turnover rate of serum albumin in man as measured by I^{131}-tagged albumin. J. clin. Invest. 1951, **30**, 1228.
14. Rothschild, M. A., Bauman, A., Yalow, R. S., and Berson, S. A. Tissue distribution of I^{131} labeled human serum albumin following intravenous administration. J. clin. Invest. 1955, **34**, 1354.
15. Gitlin, D., Landing, B. H., and Whipple, A. The localization of homologous plasma proteins in the tissues of young human beings as demonstrated with fluorescent anti-bodies. J. exp. Med. 1953, **97**, 163.
16. Eckhardt, R. D., Lewis, J. H., Murphy, T. L., Batchelor, W. H., and Davidson, C. S. Chemical, clinical and immunologic studies on the products of human plasma fractionation. XXXIV. Comparative studies of the nutritive value of orally and intravenously administered human serum albumin in man. J. clin. Invest. 1948, **27**, 119.
17. Schwartz, E. The effect of thyroid hormone upon the degradation rate and miscible pool of radioiodinated human serum albumin in myxedema. J. Lab. clin. Med. 1955, **45**, 340.
18. Dixon, F. J., Talmage, D. W., Maurer, P. H., and Deichmiller, M. The half-life of homologous gamma globulin (antibody) in several species. J. exp. Med. 1952, **96**, 313.
19. Rothschild, M. A., Bauman, A., Yalow, R. S., and Berson, S. A. The effect of large doses of desiccated thyroid on the distribution and metabolism of albumin-I^{131} in euthyroid subjects. J. clin. Invest. 1957, **36**, 422.
20. Steinbock, H. L., and Tarver, H. Plasma protein. V. The effect of the protein content of the diet on turnover. J. biol. Chem. 1954, **209**, 127.
21. Ord, W. M., and White, E. Clinical remarks on certain changes observed in the urine in myxedema after the administration of glycerine extract of thyroid gland. Brit. med. J. 1893, **2**, 217.
22. Boothby, W. M., Sandiford, I., Sandiford, K., and Slosse, J. The effect of thyroxin on the respiratory and nitrogenous metabolism of normal and myxedematous subjects. I. A method of studying the reserve or deposit protein with a preliminary report on the results obtained. Ergebn. Physiol. 1925, **24**, 728.
23. Byrom, F. B. The nature of myxoedema. Clin. Sci. 1934, **1**, 273.
24. Hoberman, H. D., and Graff, J. Influence of thyroxine on the metabolism of amino acids and proteins during fasting. Yale J. Biol. Med. 1950, **23**, 195.
25. Lange, K. Capillary permeability in myxedema. Amer. J. med. Sci. 1944, **208**, 5.
26. White, B. V., and Jones, C. M. The rate of filtration through the capillary walls as measured by the pressure plethysmograph. Observations on control subjects and on patients with intrahepatic disease, thyrotoxicosis, and myxedema. J. clin. Invest. 1939, **18**, 73.
27. Pappenheimer, J. R., and Soto-Rivera, A. Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hind limbs of cats and dogs. Amer. J. Physiol. 1948, **152**, 471.
28. Zondek, H., Michael, M., and Kaatz, A. The capillaries in myxedema. Amer. J. med. Sci. 1941, **202**, 435.
29. Landis, E. M., Jonas, L., Angevine, M., and Erb, W. The passage of fluid and protein through the human capillary wall during venous congestion. J. clin. Invest. 1932, **11**, 717.
30. Stead, E. A., Jr., and Warren, J. V. The protein content of the extracellular fluid in normal subjects after venous congestion and in patients with cardiac failure, anoxemia, and fever. J. clin. Invest. 1944, **23**, 283.
31. Landis, E. M. Micro-injection studies of capillary blood pressure in human skin. Heart 1930, **15**, 209.
32. Wells, H. S., Youmans, J. B., and Miller, D. G., Jr. A formula and nomogram for the estimation of the osmotic pressure of colloids from the albumin and total protein concentrations of human blood sera. J. clin. Invest. 1933, **12**, 1103.
33. Landis, E. M. Micro-injection studies of capillary permeability. III. The effect of lack of oxygen on the permeability of the capillary wall to fluid and to the plasma proteins. Amer. J. Physiol. 1928, **83**, 528.
34. Thompson, W. O. Studies in blood volume. I. The blood volume in myxedema, with a comparison of plasma volume changes in myxedema and cardiac edema. J. clin. Invest., 1926, **2**, 477.

MECHANISM OF INTRINSIC FACTOR ACTION IN EVERTED SACS OF RAT SMALL INTESTINE *

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Three decades have passed since Castle (1) and his associates demonstrated the existence of intrinsic factor, yet knowledge of its mechanism of action is still so limited that in 1958 two workers felt justified in concluding there was no such thing as intrinsic factor (2). In prior studies (3, 4), a mechanism involved in the enhanced uptake of vitamin B_{12} by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC) was demonstrated. This mechanism appeared to be dependent on the presence on liver slices of receptors for intrinsic factor, which in the presence of calcium would "take up" nonmetabolically either free intrinsic factor or intrinsic factor bound to vitamin B_{12}. The present studies indicate the same mechanism may operate in the rat small intestine. A preliminary report of this work has been presented (5).

MATERIALS AND METHODS

The basic protocol for each experiment was that previously used in studies with rat liver slices (4). It consisted of two successive one hour incubations of everted sacs (6) of rat small intestine at 6° C. in air; the first with 0.5 mg. HIFC [1] in the Krebs-Ringer-Tris (4) incubation medium, the second with 0.01 μg. vitamin B_{12}–Co^{60} added. Controls were run identically, except for exclusion of HIFC. Radioactivity retained in the mucosa of the everted sacs after three washings was determined in a well-type scintillation counter. Each sample was counted for a minimum of 10,000 counts. Radioactivity was expressed as counts per minute per 10 cm. of small intestine length.

The sacs were prepared as follows: A healthy adult female rat of the Wistar strain was sacrificed. The small intestine was immediately removed by transecting the gut at the duodenal end, gently pulling it free of its mesenteric attachment, and transecting it again at the distal end of the ileum. The proximal 6 cm. was discarded. A No. 6 Cournand single lumen woven cardiac catheter [2] was slipped through the lumen of the small intestine, starting at the ileal end. When the catheter tip appeared at the duodenal end, a ligature was placed tightly over the ileal end to prevent passage of the base of the catheter. Continuous gentle traction on the tip of the catheter then brought about complete eversion of the small intestine, which was immediately placed in 0.9 per cent NaCl at 0° C. Adherent intestinal content was removed by washing in three changes of ice-cold 0.9 per cent NaCl. Starting at the duodenal end, the intestine was cut into 20 segments of equal length. The ends of each segment were closed with Schwartz straight temporary arterial intracranial clamps.[3] No detectable adsorption of vitamin B_{12}–Co^{60} onto these clamps was noted after the usual washings. The segments were placed sequentially into four beakers containing the standard incubation medium, so that segments numbered 1, 5, 9, 13 and 17 were in beaker No. 1, and so forth. Although only one experiment of each type is reported, each experiment was repeated at least twice for verification.

RESULTS

Simultaneous incubation. Sacs incubated in the standard medium with both HIFC and vitamin B_{12}–Co^{60} added simultaneously showed no enhancement of vitamin B_{12}–uptake due to HIFC (Experiments 1 and 2, Table I). This was true at six different concentrations of HIFC, including the concentration (25 μg.) which produced a more than threefold enhancement of vitamin B_{12}–Co^{60} uptake in the simultaneous incubation system with rat liver slices (4).

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[1] Kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen, Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y. This preparation (No. WES671A) was clinically active in a daily oral dose of 5 mg., as determined by urinary excretion studies of the Schilling type (7).

[2] Purchased from United States Catheter and Instrument Corp., 334 Bay Street, Glen Falls, N. Y.

[3] Purchased from Storz Instrument Co., 4570 Audubon Avenue, St. Louis 10, Mo.

TABLE I

Effect of hog intrinsic factor concentrate (HIFC) on vitamin B_{12}–Co^{60} uptake by everted sacs of rat small intestine under various experimental conditions

Experiment	Cpm/10 cm. of intestine	
	Total	Excess over control
1. Simultaneous incubation		
Control	123	0
500 μg. HIFC	104	−19
50 μg. HIFC	88	−35
5 μg. HIFC	124	1
2. Simultaneous incubation		
Control	95	0
37.5 μg. HIFC	89	−6
25.0 μg. HIFC	88	−7
12.5 μg. HIFC	110	15
3. Standard conditions*		
Control	126	0
500 μg. HIFC	259	133
50 μg. HIFC	138	2
5 μg. HIFC	129	3
4. High HIFC concentrations*		
Control	103	0
50,000 μg. HIFC	94	−9
5,000 μg. HIFC	130	27
500 μg. HIFC	449	346
5. Calcium effect*		
Standard medium		
Control	196	0
HIFC	874	678
Standard medium with sodium replacing calcium		
Control	301	0
HIFC	680	379
6. EDTA† effect*		
3rd incubation without EDTA		
Control	98	0
HIFC	510	412
3rd incubation with EDTA		
Control	103	0
HIFC	294	191
7. 6° C. *vs.* 37° C.*		
Standard incubation (6° C.)		
Control	133	0
HIFC	597	464
Incubation at 37° C.		
Control	466	0
HIFC	457	−9
8. Effect of heated HIFC*		
Control	95	0
HIFC	502	407
Heated HIFC	106	11
9. Effect of 5,6-dimethylbenzimidazole*		
Standard incubation		
Control	124	0
HIFC	469	345
5,6-dimethylbenzimidazole added		
Control	117	0
HIFC	497	380
10. Effect of $CoCl_2$*		
Standard incubation		
Control	106	0
HIFC	493	387
$CoCl_2$ added		
Control	103	0
HIFC	509	406
11. 5,6-dimethylbenzimidazole and $CoCl_2$*		
Standard incubation		
Control	115	0
HIFC	498	383
5,6-dimethylbenzimidazole and $CoCl_2$ added		
Control	101	0
HIFC	514	413
12. Effect of pH 1.85*		
Standard incubation		
Control	137	0
HIFC	430	293
First incubation at pH 1.85		
Control	141	0
HIFC	302	161
13. Effect of pH 1.85*		
Standard incubation		
Control	107	0
HIFC	393	286
Second incubation at pH 1.85		
Control	109	0
HIFC	227	118

* Sequential incubation, *i.e.*, two successive one-hour incubations: the first with 0.5 mg. HIFC (unless otherwise stated) in the incubation medium, the second with 0.01 μg. vitamin B_{12}–Co^{60} added.

† Disodium ethylenediaminetetraacetate dihydrate.

Sequential incubation. Sacs incubated first with 0.5 mg. of HIFC and then with vitamin B_{12}–Co^{60} invariably showed a significant enhancement of the uptake of vitamin B_{12}–Co^{60} (Table I). However, no clear enhancement was observed in the sequential incubation system when 10-fold or 100-fold higher or lower concentrations of HIFC were used (Experiments 3 and 4, Table I).

Effect of calcium-free medium. The enhancement of uptake produced by HIFC was markedly reduced in the absence of calcium (Experiment 5, Table I). The marked increase in the uptake of vitamin B_{12}–Co^{60} by control tissues in the

absence of calcium may be due to increased cell permeability. The same phenomenon has been observed in studies with rat liver slices (3).

Effect of disodium ethylenediaminetetraacetate dihydrate (EDTA). After performance of the standard experimental procedure, sacs were incubated for a third hour in 10 ml. of Krebs-Ringer-Tris to which was added 2 ml. of 0.9 per cent NaCl either without disodium ethylenediaminetetraacetate dihydrate (EDTA) or containing 2 Gm. of the material per 100 ml. As noted in Table I, Experiment 6, the addition of EDTA brought about no change in control values, but markedly reduced the amount of vitamin B_{12}–Co^{60} retained by sacs previously incubated in the presence of added HIFC.

Temperature. When the standard two incubations were performed at 37° C. instead of 6° C., the intestinal sac mucosa became pale and friable; microscopic examination [4] demonstrated destruction of the mucosa. Such sacs showed very high control uptake and no enhancement by HIFC (Experiment 7, Table I).

Heating HIFC. The addition of HIFC which had been heated in a water bath at 100° C. for 15 minutes resulted in no enhancement of uptake of vitamin B_{12}–Co^{60} (Experiment 8, Table I).

Effect of 5,6-dimethylbenzimidazole [5] and $CoCl_2$. Neither 5,6-dimethylbenzimidazole nor $CoCl_2$ interfered with the uptake of vitamin B_{12}–Co^{60} by everted sacs of rat small intestine either without or with preincubation with HIFC (Experiments 9, 10 and 11, Table I). This was true when either or both agents were added to the incubation medium just before the start of the second incubation and prior to the addition of vitamin B_{12}–Co^{60}. The amount of 5,6-dimethylbenzimidazole added was 1 μg. dissolved in 1 ml. of 0.9 per cent NaCl (producing a ratio of 100 units of 5,6-dimethylbenzimidazole to 1 unit of vitamin B_{12}–Co^{60} by weight). The amount of $CoCl_2$ added was 1 ml. of 0.11 M solution.

Effect of incubation at pH 1.85. Performance of either the first or the second incubation at pH 1.85 instead of pH 7.5 had no effect on uptake of control sacs but reduced the enhancement of vitamin B_{12}–Co^{60} uptake normally observed after incubation with HIFC (Experiments 12 and 13, Table I).

[4] Microscopic preparations made through the courtesy of Dr. H. M. Zimmerman, Department of Pathology, Montefiore Hospital.

[5] Kindly provided by Dr. K. Folkers, Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

DISCUSSION

A previous study (8) demonstrated that HIFC enhanced vitamin B_{12} uptake by open segments of rat small intestine. Since both the mucosal and serosal surfaces were exposed to the incubation medium, such open segments were not suited to study of the mechanism of intrinsic factor action on the mucosal surface; results were complicated by serosal surface vitamin B_{12} uptake which markedly elevated control values. This problem was eliminated in the present study by using closed everted sacs of rat small intestine (6). Only the mucosal surface of such sacs is exposed to the incubation medium.

The results of this study with everted sacs of rat small intestine are strikingly similar to the results of prior studies with rat liver slices (3, 4). In both cases, enhancement of vitamin B_{12}–Co^{60} uptake by HIFC is most marked in a sequential incubation system (*i.e.,* a system of incubation with HIFC alone followed by a second incubation with vitamin B_{12}–Co^{60} alone). In both cases this enhancement is observable in the cold, is maximal in the presence of adequate calcium, and is reversible to a significant degree by EDTA. In a calcium-free medium, no enhancement of vitamin B_{12} uptake attributable to intrinsic factor occurs on liver slices, but a definite enhancement is still observable with everted sacs of small intestine. This may be due to difficulty in washing the everted sacs free of calcium prior to incubation.

That the increase in the mucosal uptake of vitamin B_{12}–Co^{60} after preincubation with HIFC is due to intrinsic factor is suggested by the facts that: *1*) the increase is abolished by preheating the HIFC at 100° C. for 15 minutes, a process which destroys intrinsic factor activity (9); *2*) performance of either incubation at pH 1.85 markedly reduces the enhancement of the uptake of vitamin B_{12}–Co^{60} otherwise observable (and no enhancement occurs if both incubations are performed at pH 1.85 [10]). (At this pH the effect of intrinsic factor in enhancing vitamin B_{12} absorption from the rat intestine *in vivo* is

reduced [11]. It is not yet clear how much of the reduction is due to intrinsic factor instability at pH 1.85, and how much may be due to instability of vitamin B_{12}-intrinsic factor or intrinsic factor-receptor bonds at this pH.); *3*) 5,6-dimethylbenzimidazole, either alone or in combination with $CoCl_2$, does not affect the binding by the system of vitamin B_{12}–Co^{60}, as it does not interfere with intrinsic factor action in human subjects (12). This latter point may suggest that in the sac formed by the everted small intestine of the rat, as in the small intestine of the human subject, the 5,6-dimethylbenzimidazole portion of cyanocobalamin, as well as the cobalt portion, may not be involved in the interaction with intrinsic factor. Of course, although these substances *as such* did not interfere with the uptake of vitamin B_{12}, it is possible that *as portions of the cyanocobalamin molecule* they may be involved in the interaction with intrinsic factor.

These findings would suggest that the mechanism of intrinsic factor action may be the same in the small intestine as in the liver slice. It may be postulated that intrinsic factor has the ability on the one hand to bind vitamin B_{12} (9) and on the other to attach to liver or intestine receptors in the presence of calcium. Possession of these two properties simultaneously may be unique to intrinsic factor. Heparin, chondroitin sulfate and orosomucoid do not increase vitamin B_{12}–Co^{60} uptake by everted sacs of rat small intestine (10), as they did not increase vitamin B_{12}–Co^{60} uptake by rat liver slices (3, 4). This would indicate that enhancement of vitamin B_{12} uptake is not a nonspecific property of any mucopolysaccharide or mucoprotein.

The concept of an intestine receptor for intrinsic factor fits all the facts which led Wallerstein, Harris, Schilling and Castle (13) to "infer that the fundamental action of intrinsic factor is on a superficially located hypothetical structure or chemical process in the wall of the upper small intestine"; and led Glass, Boyd and Stephanson (14) to postulate the existence of an "intestinal vitamin B_{12} acceptor." In addition, it allows of explanation of the control of absorption of physiologic amounts of vitamin B_{12} in terms of a single mechanism at the cell surface, rather than of separate mechanisms at the mucosal membrane and intramurally.

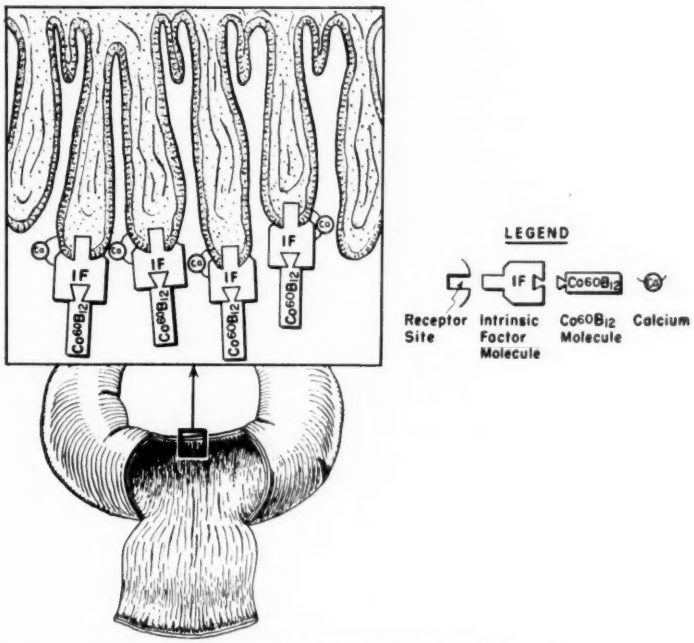

FIG. 1. OPTIMAL INTRINSIC FACTOR CONCENTRATION

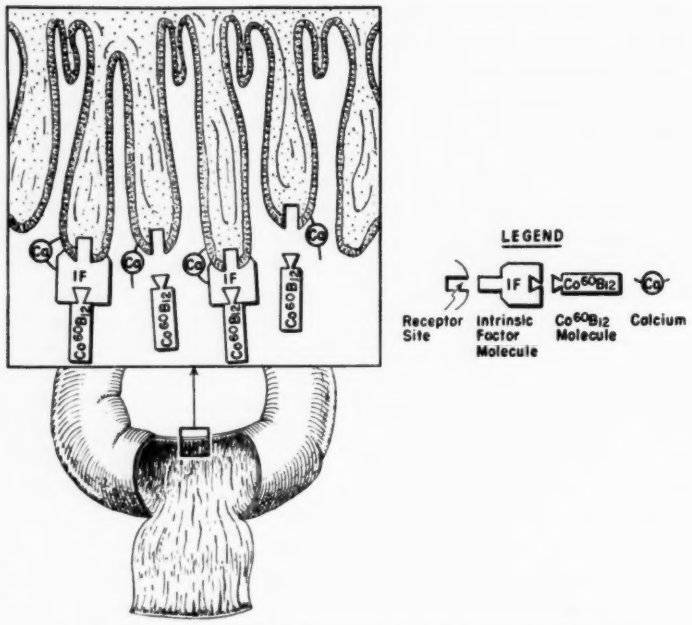

FIG. 2. HALF OPTIMAL INTRINSIC FACTOR CONCENTRATION

This formulation of the role of intrinsic factor in the first step in the absorption of physiologic amounts of vitamin B_{12} is schematically depicted in Figures 1, 2 and 3. Figure 1 pictorially represents the ideal situation: an identical number of receptor sites, intrinsic factor molecules and vitamin B_{12}–Co^{60} molecules. Maximal absorption of vitamin B_{12} would occur in this optimal situation. In Figure 2, the decreased uptake of vitamin B_{12} in the presence of an inadequate amount of intrinsic factor is portrayed. Figure 3 indicates the way the hypothesis of "receptor sites for intrinsic factor" may be used to explain the observed phenomenon *in vivo* (15–17) of decreased vitamin B_{12} absorption in the presence of large amounts of intrinsic factor concentrates. When there are more molecules of intrinsic factor than there are receptor sites or molecules of vitamin B_{12}, a portion of the receptor sites will be occupied by intrinsic factor molecules which are not binding vitamin B_{12}. Since the excess of intrinsic factor saturates the receptor sites, that vitamin B_{12} which remains in the lumen of the intestine, bound to intrinsic factor which has no available receptor site to go to, is lost in the feces.

It must be emphasized that alternative explanations for the inhibitory effect of large amounts of HIFC must be considered. These include: *1*) possible contamination of HIFC preparations with nonintrinsic factor vitamin B_{12} binding materials; *2*) possible presence in HIFC preparations of inhibitors of intrinsic factor action. Neither of these possibilities can be ruled out until intrinsic factor is isolated in pure form. The most potent HIFC preparation available (No. WES655)[6] inhibited vitamin B_{12} uptake by everted sacs of rat small intestine when used in large quantity (10), but even this preparation can not be considered free of contaminants.

Whether this schema is an accurate depiction of the first stage of vitamin B_{12} absorption *in vivo* remains to be determined. Preliminary observations, using sodium phytate to "tie up" calcium in the intestine of human subjects, suggest that the mechanism of intrinsic factor action may indeed be calcium-dependent (18). In other stud-

[6] Kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen. This preparation was clinically active in a daily oral dose of 1 mg., as determined by urinary excretion studies of the Schilling type (7).

ies of the relationship of results in the *in vitro* system here reported to those *in vivo,* it was found that the maximal effect of HIFC occurs in those everted sacs from the middle two quarters of the small intestine (10); the maximal effect of intrinsic factor in the living rat is also in the middle quarters of the small intestine (19). The reason for discarding the proximal 6 cm. of small intestine in all the everted sac experiments reported here was that no effect of HIFC was demonstrable in that portion of the gut.

A further parallel of the *in vitro* system with the *in vivo* situation is the demonstration of two separate mechanisms of vitamin B_{12} uptake in both. One mechanism is intrinsic factor-dependent, and plays the major role in absorption of physiologic amounts of vitamin B_{12} (20, 21). It is this mechanism with which this report is mainly concerned. The other mechanism appears to be a "mass action phenomenon," possibly diffusion, and is operative in bringing about the absorption of small amounts of vitamin B_{12}. This latter mechanism is most readily observable in the human subject after the oral administration of supraphysiologic amounts of vitamin B_{12} (20, 21). In the everted sac of rat small intestine, it may be this mechanism which produces the vitamin B_{12} uptake observed in controls.

The failure to observe enhanced vitamin B_{12}–Co^{60} uptake by everted sacs of rat small intestine after incubation with large amounts of HIFC, or after simultaneous incubation with amounts of HIFC which produced marked enhancement of liver slice vitamin B_{12} uptake, is not yet explained. The former may be explainable by "trapping" of large amounts of HIFC between the intestinal villi, to be released during the subsequent incubation with vitamin B_{12}–Co^{60} and then to compete for the vitamin with the intrinsic factor on receptor sites. The latter may be explainable by the presence of fewer free receptors on the intestinal wall than on the liver slice. This is suggested by the fact that enhancement of vitamin B_{12} uptake by HIFC is not as many times more than control values for intestine sacs as for liver slices. It is possible that many receptor sites on the rat intestinal wall are occupied by rat intrinsic factor binding vitamin B_{12} taken in the rat's diet prior to sacrifice. Studies with everted sacs of small intestine from

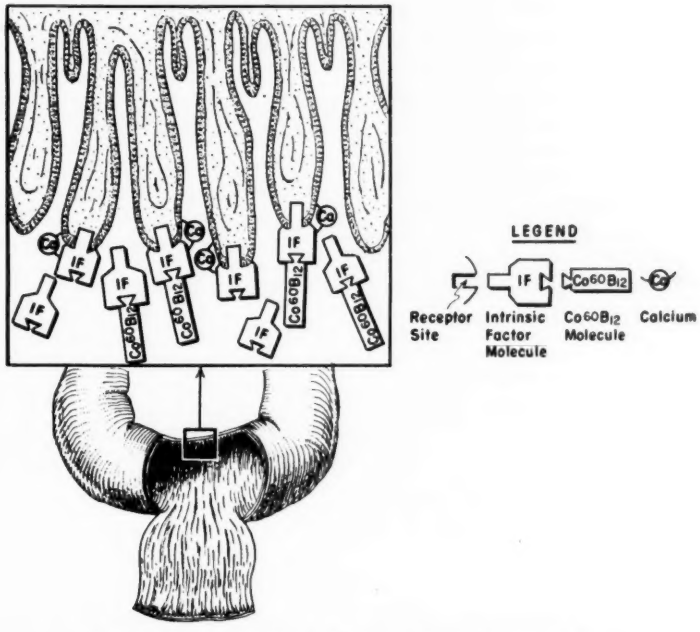

FIG. 3. TWICE OPTIMAL INTRINSIC FACTOR CONCENTRATION

gastrectomized rats may provide useful information in this connection.

Finally, the demonstration that hog intrinsic factor concentrate will enhance vitamin B_{12} uptake by rat small intestine further weakens the indirectly (22) and directly (17) attacked concept of species specificity of intrinsic factor as a phenomenon of major importance. Data previously reported (8) would suggest the interesting speculation that perhaps an organ specificity may exist for intrinsic factor and may be of greater significance than the species specificity.

SUMMARY

Vitamin B_{12} uptake by everted sacs of rat small intestine is enhanced markedly after preincubation with hog intrinsic factor concentrate (HIFC). This enhancement occurs at 6° C., is maximal in a calcium-containing incubation medium, and is reversible to a significant degree by disodium ethylenediaminetetraacetate dihydrate (EDTA). Procedures which interfere with intrinsic factor action, such as heating at 100° C. or incubating at pH 1.85, eliminate or reduce this enhancement.

The close similarity of these findings to those previously obtained with rat liver slices is discussed. The hypothesis is presented that the first step in the absorption of physiologic amounts of vitamin B_{12} by the small intestine is binding of the vitamin to intrinsic factor and binding of intrinsic factor to intestine receptors (in the presence of calcium). It is suggested that possession of these two binding properties simultaneously may be unique to intrinsic factor.

The uptake of vitamin B_{12} by everted sacs of rat small intestine in the absence of HIFC may be the *in vitro* parallel of a nonintrinsic factor-dependent mechanism of vitamin B_{12} absorption observed *in vivo* and operative primarily in the presence of supraphysiologic amounts of vitamin B_{12}.

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REFERENCES

1. Castle, W. B. Medical progress; development of knowledge concerning the gastric intrinsic factor and its relation to pernicious anemia. New Engl. J. Med. 1953, **249**, 603.
2. Heathcote, J. G., and Mooney, F. S. The oral treatment of pernicious anaemia. Lancet 1958, **1**, 982.
3. Herbert, V. Studies of the mechanism of the effect of hog intrinsic factor concentrate on the uptake of vitamin B_{12} by rat liver slices. J. clin. Invest. 1958, **37**, 646.
4. Herbert, V. Development of a possible *in vitro* assay for intrinsic factor. Proc. Soc. exp. Biol. (N. Y.) 1958, **97**, 668.
5. Herbert, V. Mechanism of intrinsic factor action in the isolated rat small intestine (abstract). J. clin. Invest. 1958, **37**, 901.
6. Wilson, T. H., and Wiseman, G. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 1954, **123**, 116.
7. Ellenbogen, L., Williams, W. L., Rabiner, S. F., and Lichtman, H. C. An improved urinary excretion test as an assay for intrinsic factor. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 357.
8. Herbert, V., and Spaet, T. H. Distribution of "intrinsic factor" activity. Amer. J. Physiol. 1958, **195**, 194.
9. Grasbeck, R. Studies on the vitamin B_{12}-binding principle and other biocolloids of human gastric juice. Acta med. scand. 1956, Suppl. **314**.
10. Herbert, V. Unpublished observations.
11. Niewig, H. O., Shen, S. C., and Castle, W. B. Mechanism of intrinsic factor action in the gastrectomized rat. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 223.
12. Bunge, M. B., Schloesser, L. L., and Schilling, R. F. Intrinsic factor studies. IV. Selective absorption and binding of cyanocobalamin by gastric juice in the presence of excess pseudovitamin B_{12} or 5,6-dimethylbenzimidazole. J. Lab. clin. Med. 1956, **48**, 735.
13. Wallerstein, R. O., Harris, J. W., Schilling, R. F., and Castle, W. B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. XV. Hematopoietic effects of simultaneous intravenous and of simultaneous or serial oral administration of intrinsic factor and vitamin B_{12}. J. Lab. clin. Med. 1953, **41**, 363.
14. Glass, G. B. J., Boyd, L. J., and Stephanson, L. Intestinal absorption of vitamin B_{12} in humans as studied by isotope technic. Proc. Soc. exp. Biol. (N. Y.) 1954, **86**, 522.
15. Glass, G. B. J., Boyd, L. J., Stephanson, L., and Jones, E. L. Metabolic interrelations between intrinsic factor and vit. B_{12}. III. B_{12} absorption at varied intrinsic factor doses. Proc. Soc. exp. Biol. (N. Y.) 1955, **88**, 1.
16. Callender, S. T., and Evans, J. R. Observations on the relationship of intrinsic factor to the absorption of labelled vitamin B_{12} from the intestine. Clin. Sci. 1955, **14**, 387.
17. Taylor, K. B., Mallett, B. J., Witts, L. J., and Taylor,

W. H. Observations on vitamin B_{12} absorption in the rat. Brit. J. Haemat. 1958, **4**, 63.

18. Herbert, V. Studies of intrinsic factor mechanism of action. Vitamin B_{12} Symposium, New York Medical College, April 11, 1958. In press.
19. Reynell, P. C., Spray, G. H., and Taylor, K. B. The site of absorption of vitamin B_{12} in the rat. Clin. Sci. 1957, **16**, 663.
20. Ross, G. I. M., Mollin, D. L., Cox, E. V., and Ungley, C. C. Hematologic responses and concentration of vitamin B_{12} in serum and urine following oral administration of vitamin B_{12} without intrinsic factor. Blood 1954, **9**, 473.
21. Doscherholmen, A., and Hagen, P. S. A dual mechanism of vitamin B_{12} plasma absorption. J. clin. Invest. 1957, **36**, 1551.
22. Abels, J., Woldring, M. G., Vegter, J. J. M., and Niewig, H. O. Effect of rat intrinsic factor on vitamin B_{12} absorption in pernicious anemia. Science 1957, **126**, 558.

THE CHARACTERISTICS AND SIMILARITY OF PRIMARY AND SECONDARY PERISTALSIS IN THE ESOPHAGUS *

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Primary and secondary peristalsis in the esophagus are believed to differ in their modes of initiation and propagation. Since primary peristalsis is, by definition, initiated by a swallow and secondary peristalsis set off by esophageal distention, their difference with respect to initiation is an *a priori* matter. Recent studies (1, 2), however, challenge the concept that a clear distinction exists as far as propagation is concerned.

Classically, primary peristalsis is said to be centrally integrated—that is, the afferent impulse released by swallowing stimulates a medullary center which thereupon, without further afferent stimulation, activates in succession oral, pharyngeal and esophageal musculature (3). Secondary peristalsis was contrasted by Meltzer and Auer (4) as follows: "This peristaltic movement, however, differs from the normal peristalsis after deglutition in the essential point that it does not progress in the lower section of the oesophagus if the latter be divided transversely at some place. That shows that this form of peristalsis depends upon consecutive stimulations of the mucous membrane of the oesophagus and consists of a chain of local reflexes."

The nature of secondary peristalsis has generally been investigated by inserting a bolus into the esophagus and analyzing the transport phenomena elicited. This technique, however, has obvious limitations since the presense of a bolus could elicit local stimuli and reflexes on moving downstream. Under these circumstances, it is impossible to tell whether secondary peristalsis, once elicited, would similarly pass down the esophagus were no bolus present. With modern manometric devices this question can now be solved, for the propagation of secondary peristalsis can be analyzed without using liquid, semisolid or solid boluses that might stimulate local reflexes. We have therefore initiated secondary peristalsis in the human esophagus by local distention, have studied the resultant inhibitory and motor phenomena by the technique of intraluminal manometry, and have compared the effects produced with those obtained when primary peristalsis is induced by swallowing.

METHODS

A tube assembly, as used in previous studies (5) and consisting of three open-tipped and water-filled polyvinyl tubes, was passed via the nose into the esophagus of healthy volunteers. The tips were spaced 3 cm. apart. Pressure changes at the tips were transmitted through the water columns to Sanborn electromanometers and recorded. An external pneumograph identified respiratory excursions on one channel. All recordings of intraluminal esophageal pressure were made with the subject supine and the manometers leveled at the posterior axillary line. The high pressure zone which is found in the distal esophagus and ascribed to the lower esophageal sphincter (6) was located by first placing the distal recording tip in the stomach and then withdrawing it centimeter by centimeter.

A balloon of 40 ml. capacity was attached to a Levin tube, passed into the esophagus, and the proximal end of the tube firmly fixed. Radiologic localization of the balloon and of the radiopaque pressure recording tips was carried out in all cases. The balloon was inflated by several techniques. Momentary or prolonged inflation was carried out by injecting an absolute amount of air rapidly with a syringe, holding the air in the balloon for the desired period of time, and then aspirating the air quickly. To achieve a more physiologic distention stimulus which would not move but would yield to esophageal contraction isobaric inflation of the balloon was used. In this system, which has been used extensively to record motility (7, 8), the balloon is inflated under moderate, approximately constant pressure (6 to 12 cm. water) produced by the difference between water levels of two

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chambers in a damped kymographic system (9). The amount of air allowed in the balloon is determined by the esophageal reaction: Once the balloon has reached a certain size, the esophagus contracts and expels air back into the external system. During the various techniques of inflation used, the balloon was also connected to an electromanometer and writing channel in order to provide for accurate recording of times of inflation and deflation, or of esophageal contraction or relaxation in the area of the balloon.

RESULTS

1. *Effect of momentary distention on esophageal motility*

Rapid inflation and deflation of the fixed balloon was carried out 115 times in 11 subjects. The balloon was held inflated for a mean time of 3.7 seconds. In 55 tests (47 per cent), progressive pressure waves were produced and recorded as passing sequentially over tips placed in the esophagus distal to the ballon (Figure 1A). No swallowing movements preceded the appearance of these waves. A nonprogressive pressure change appearing in only one tip, called a "spasm," was elicited in an additional seven tests. The amount of air used for inflation of the balloon varied from 5 to 40 ml. with a mean of 21 ml. No correlation was found between the amounts of air used and the incidence of either progressive or nonprogressive positive pressure responses. Substernal pain was occasionally produced when 35 to 40 ml. was injected but did not appear to affect the results. In those tests in which a moving pressure wave was elicited by esophageal distention, the interval between beginning of balloon inflation and the start of the pressure rise at the tip nearest the balloon was 5.5 seconds; the interval between the beginning of balloon deflation and the start of pressure rise at the nearest tip, 2.1 seconds. Since the duration of inflation averaged 3.7 seconds, it appears that initiation of the moving pressure wave in this experimental design was closely related to deflation. The speed of progression of the wave, once set off, averaged 3 cm. per second.

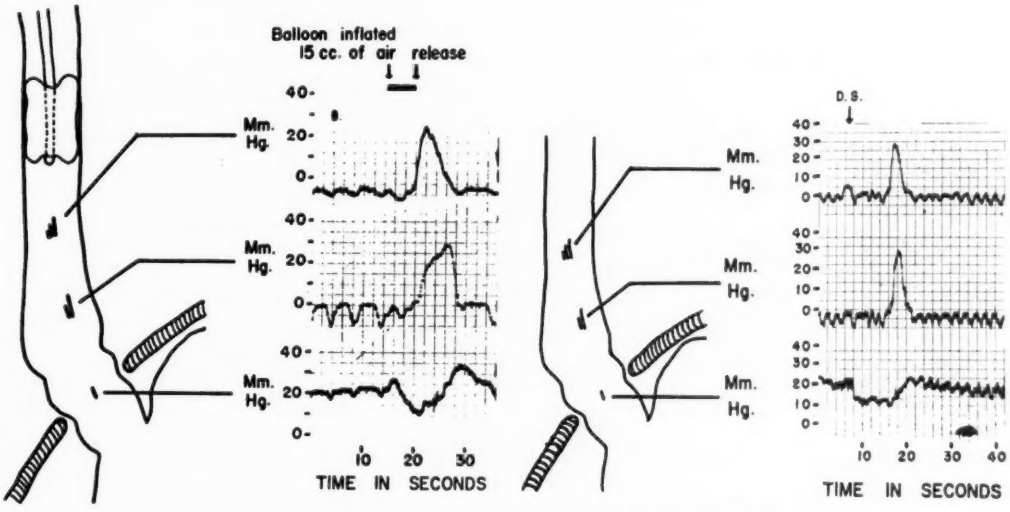

FIG. 1. COMPARISON OF SECONDARY (A) AND PRIMARY (B) ESOPHAGEAL PERISTALSIS CURVES

A. Momentary distention with an air-filled balloon fixed in the mid-esophagus produced a drop in pressure in the sphincteric zone (bottom tracing), followed by a progressive contraction recorded at two esophageal sites distal to the balloon (upper two tracings). These pressure phenomena, elicited without a swallow or moving bolus, are comparable to those characterizing primary esophageal peristalsis initiated by swallowing (Fig. 1B).

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B. The drop in pressure in the sphincteric zone (bottom tracing) and the progressive contraction in the esophagus (upper two tracings) released by a dry swallow (one containing only air and a little saliva) are demonstrated. DS indicates dry swallow.

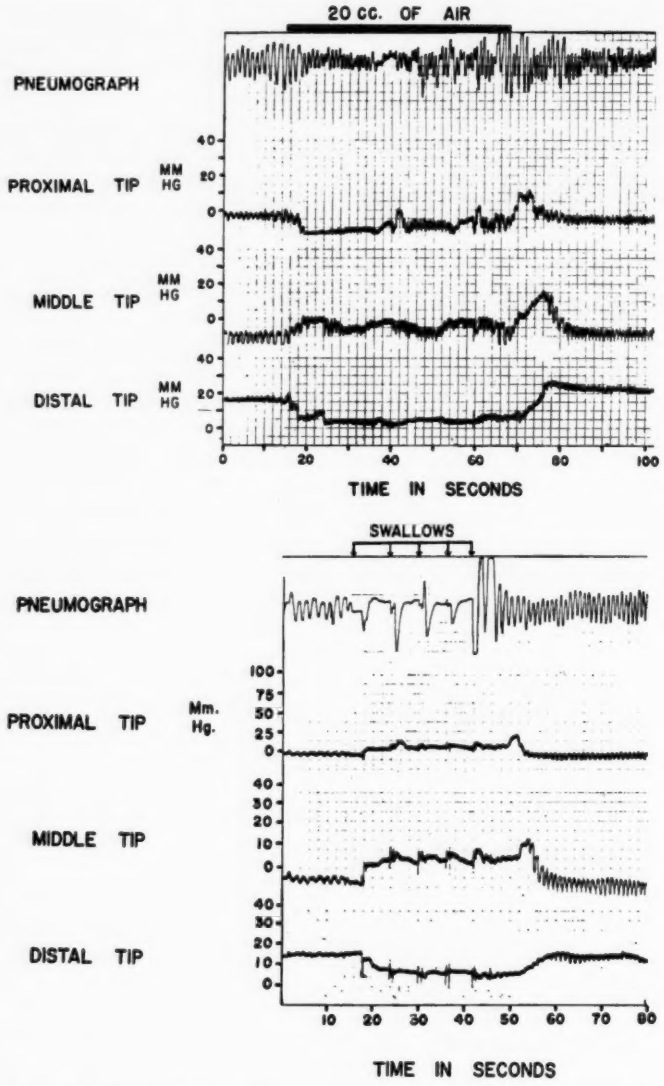

FIG. 2. PRESSURE TRACINGS DURING PROLONGED DISTENTION (A) COMPARED WITH FREQUENT SWALLOWINGS (B)

A. Prolonged distention (solid bar at top of record) of a balloon fixed in the mid-esophagus was attended by no changes in the body of the esophagus distal to the balloon (proximal and middle tips), and produced a sustained drop in intrasphincteric pressure (distal tip), maintained until the balloon was deflated. Thereafter a pressure wave passed through the lower esophagus and sphincter. The overall effect on esophageal motility is similar to that produced by rapidly repeated swallows (Fig. 2B).

B. Liquid swallows taken at approximately seven second intervals produced a sustained drop in pressure in the sphincteric zone (distal tip), and no peristaltic pressure phenomena appeared in the body of the esophagus (proximal and middle tips) during this time. The sustained rise and the occasional spikes of pressure recorded are ascribable to fluid introduced into the esophagus and to transmitted oropharyngeal pressures. After the swallowing efforts ceased, a peristaltic contraction travelled down the esophagus.

2. Effect of momentary distention on the lower esophageal sphincter

The lowermost recording tip was in the high pressure zone (lower esophageal sphincter) on 62 of the occasions when the balloon was inflated momentarily. A drop in pressure occurred 51 times, 39 being associated with the progressive pressure reactions described above. In every instance the drop in pressure in the sphincteric area preceded the rise in pressure recorded by the tip nearest the balloon (Figure 1A). The average time between the beginning of inflation and the beginning of the pressure drop was 1.5 seconds. The average time from the start of the pressure drop to deflation was 2.2 seconds. Thus the drop in pressure in the sphincteric zone seems to be related to the period of inflation when the esophagus is still distended. At the end of the period of low intrasphincteric pressure, an overshoot to levels 2 to 7 mm. Hg above resting levels occurred in 12 of the 39 tests in which progressive esophageal pressure waves were elicited by momentary distention.

In 12 instances a pressure drop in the sphincter zone occurred in the absence of any accompanying progressive pressure wave. The average amount of air used for ballon inflation in these 12 tests was 17 ml. as compared to the average amount of 21 ml. used to elicit the full sequence of an esophageal progressive pressure wave and a decrease in intrasphincteric pressure. In the group of 12 tests exhibiting only a drop in intrasphincteric pressure, this drop was less marked than in those exhibiting the full sequence, and in no case did pressures overshoot the resting levels at the completion of the low pressure interval.

No evidence of sphincteric changes was detected in the seven tests showing a "spasm" response.

3. Effects of prolonged distention

When inflation was maintained for periods of 10 seconds to 2 minutes, with no air allowed to escape from the balloon, variable patterns resulted. One effect was a drop in pressure in the high pressure zone which continued for as long as the balloon was inflated (Figure 2A). During this period no clear-cut pattern of pressure changes occurred in the body of the esophagus. After deflation, a progressive pressure wave was recorded passing over the esophagus and the sphincteric area. In this case, as was true of the response to momentary distention, the pressure drop in the high pressure zone seemed related to inflation, whereas the positive pressure sequence in the body of the esophagus seemed to be related to balloon deflation.

Prolonged distention sometimes produced no changes in the motor activity of either the esopha-

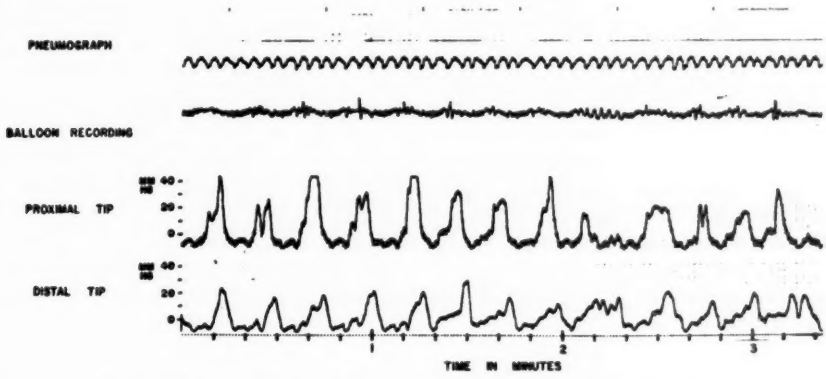

FIG. 3. EFFECT OF ISOBARIC DISTENTION ON ESOPHAGEAL CONTRACTIONS

Balloon inflation by the isobaric technique produced repeated local esophageal contractions (small spikes in balloon recording). Each contraction compressing the fixed but pliable balloon bolus was followed by a pressure wave progressing through the body of the esophagus below the balloon (proximal and distal tips).

gus or the sphincteric area. On occassion, a pressure wave was initiated and progressed down the esophagus while prolonged distention was being maintained. In these instances, the response in the esophagus distal to the balloon appeared to be preceded by a fall and then a rise of pressure within the balloon, as if the resistance of the esophageal wall to balloon distention had to diminish before a distally moving pressure wave could be elicited.

4. Effect of isobaric distention

When the esophagus in five subjects was stimulated by isobaric balloon inflation, air flowed into the balloon under the applied pressure and then flowed out in response to the resultant esophageal contraction at a rate of four to nine full cycles per minute. Each of the contractions so produced by the fixed but pliable balloon stimulus thereupon travelled down the gullet at a corresponding rate of four to nine per minute (Figure 3) and produced a moving pressure complex similar to those seen in response to momentary balloon distention. Diaphragmatic contraction, as reflected in the pneumograph, could not be correlated with the moving pressure waves.

In three cases in which the distal recording tip was in the lower esophageal sphincter zone, symmetrically spaced periods of high and low pressure were also recorded. Though not always identifiable against this fluctuating background, intrasphincteric pressure seemed to fall shortly after the beginning of each contraction about the stimulating balloon.

5. Pressure patterns produced in the esophagus above the balloon

Pressures were recorded at three sites (spaced 3 cm. apart) above the balloon during and after 21 momentary inflations in three subjects. A simultaneous increase in pressure occurred in 19 cases at two or more recording sites. In four of these, a progressive pressure wave followed the "spasm." These pressure waves had the same configuration and rate of progress as those recorded in the esophagus below a stimulating balloon. In no instance was a progressive pressure wave seen above the balloon without an antecedent "spasm."

Isobaric distention, with recording above the balloon, resulted in a different pattern from that obtained below the balloon. Instead of repetitive contractions moving aborally at a rate of four to nine per minute, "spasms," usually erratic but occasionally rhythmic at a rate of approximately nine per minute, were recorded.

DISCUSSION

The aborally moving pressure wave produced by momentary local distention of the mid-esophagus exhibits a pattern identical to that released by a "dry" swallow (Figure 1B). The configuration of the complex, its rate of progression, and the fall in pressure in the zone of the lower esophageal sphincter are common to both motor phenomena, although one is the result of a swallow, *i.e.*, primary peristalsis, and the other of local esophageal distention, *i.e.*, secondary peristalsis. No swallowing, moving bolus or respiratory effects attended the secondary wave. Thus local esophageal distention may elicit a peristaltic phenomenon in the esophagus exactly the same as that of primary peristalsis, except that manifestations of a nervous control center, such as oropharyngeal movements and respiratory interruption, are absent. Since no bolus moved downstream, propagation of the wave obviously did not depend on a bolus to set off a series of "consecutive stimulations of the mucous membrane of the oesophagus."

Although a regular pattern of secondary peristalsis could be elicited by momentary distention, the response was obtained in no more than half the tests. Creamer and Schlegel, in studying the esophageal response to distention, observed peristalsis distal to the distending balloon in only 26 per cent of tests (2). Even swallowing, however, is irregularly followed by a primary peristaltic wave, the response occurring about three-fourths of the time in the entire esophagus, and 59 per cent of the time in the lower third (10). In the distention experiments, one reason for a peristaltic response rate may lie in the stimulus itself. Certainly forcible inflation of a balloon is not comparable as a stimulus to a swallow of water and may, indeed, favor a response of inhibition over contraction.

Similar and powerful inhibitory influences participating in the control of esophageal motility

may be brought to light both by swallowing and by local esophageal distention. Either stimulus may be followed by a fall in pressure in the zone of the lower esophageal sphincter, presumably reflecting relaxation of tonically contracted muscle. As evident from the results of momentary esophageal distention, this sphincteric relaxation precedes the onset of peristaltic contraction in the body of the esophagus and may, indeed, take place without a detectable contractile response, an observation also made by others in man (2) and dogs (11). When the inhibitory phase is constantly stimulated, either by taking repetitive swallows at intervals of less than seven seconds (Figure 2B) or by maintaining forcible local distention of the esophagus, the sphincteric zone remains constantly relaxed.

Inhibitory influences in the sphincteric zone are readily demonstrable because of its high resting tonicity. In the body of the esophagus, flaccid at rest, inhibitory effects are not so easily shown but their presence may be inferred. It is evident that repetitive swallows at less than seven second intervals or prolonged balloon distention will postpone until after cessation of the stimulus the peristaltic contraction that follows a single swallow or momentary inflation of the balloon. Thus, stimuli eliciting either primary or secondary peristalsis would seem to release first a wave of inhibition that passes rapidly down the esophagus and the lower sphincter; when this phase ends, and only then, a contractile effect travels the same path. The "wave of inhibition" need not be regarded as a wave of positive inhibitory influences. It is more simply conceived of as a temporary suppression of excitatory stimuli, phasic as well as tonic.

The mere release of an inhibitory effect does not necessarily precipitate an ensuing contraction. Rather, a local contraction appears either if a rigid distending force is released, or if a moderate and yielding distending force is employed, as is the case if a liquid bolus is introduced into the esophagus or isobaric balloon distention is used. Propagation of this contraction in the form of a peristaltic wave then takes place, possibly because sensory stimuli from the area of local contraction set off a chain reaction in some nervous swallowing center controlling esophageal peristalsis. If this were the case, however, the esophageal motor response above the stimulated area might be expected also to exhibit a peristaltic pattern. Such a pattern was not recorded at all during isobaric distention of the esophagus, and after momentary distention only if a "spasm" was first elicited. A central nervous mechanism organizing secondary peristalsis should also act less erratically with respect to the incidence of peristaltic phenomena appearing in response to local esophageal stimulation. Certainly the nervous center that controls oropharyngeal swallowing mechanisms is distinguished by the constancy and reproducibility of its sequence (12).

The alternate possibility is that the local stimulus sets off a contractile impulse which passes down the esophagus independently of a swallowing center. Responsible pathways would include vago-vagal reflexes, myenteric plexus activity, and perhaps even muscle-to-muscle transmission. The participation of vago-vagal reflexes is indicated not only by impairment of secondary peristalsis achieved by high vagal section in animals (13), but also by Andrew's findings in rats that the very passage of a contraction, without any bolus, is sufficient to release afferent impulses (14).

Secondary peristalsis may be defined as a wave stimulated by transient local distention of the esophagus, characterized by sequential phenomena of inhibition and excitation, and depending for its propagation neither on a swallowing center nor a moving bolus. Except for its method of stimulation and its apparent independence of a swallowing center, secondary peristalsis is thus identical in its manifestations to primary peristalsis. The question then arises: Are primary and secondary peristalsis really different? Is not primary peristalsis merely a peristaltic phenomenon precipitated when either a bolus or a contraction is passed from the pharynx into the esophagus? A number of observations made in moderately anesthetized animals would indicate that primary and secondary peristalsis differ in their modes of esophageal propagation (1, 4, 13), but these observations are not unequivocally conclusive. The studies here reported and the erratic behavior of primary as well as secondary peristalsis suggest that the basic mechanisms underlying both types of esophageal peristalsis are essentially the same.

SUMMARY

Esophageal peristalsis is traditionally separated into two types: primary, set off by swallowing and propagated by stimuli emanating from a medullary swallowing center; and secondary, initiated by esophageal distention and propagated by sequential short reflex arcs stimulated by the moving bolus. The studies underlying this concept of secondary peristalsis made use, however, of a moving bolus, a circumstance which makes it impossible to tell whether secondary peristalsis, once elicited, would similarly pass down the esophagus were no bolus present.

In our studies, secondary peristalsis was initiated by distending an esophageal balloon that was fixed, and by measuring the resultant motor and inhibitory phenomena above and below the balloon with the aid of manometric devices too small to affect motility. When the balloon was distended momentarily, the high resting pressure in the zone of the lower esophageal sphincter fell, and a pressure wave travelled down the esophagus. The total sequence was identical to that produced when primary peristalsis is initiated by a dry swallow. By varying the method of balloon inflation, motor and inhibitory phenomena identical to those produced by rapidly or slowly repeated swallows were obtained. Above the distending balloon, nonpropulsive contractions were the usual reactions to balloon distention. No evidence was obtained to indicate that a nervous swallowing center was controlling the secondary peristaltic phenomena elicited.

The propagation of secondary peristalsis thus does not require a moving bolus to stimulate sequential motor reflexes, nor does it appear to be dominated by a swallowing center. The orderly progress of secondary peristalsis presumably depends, therefore, on impulses released by the contraction itself and transmitted via vago-vagal, myenteric plexus, and perhaps intermuscular activity.

Primary and secondary peristalsis are remarkably similar in their manometric manifestations, their independence of an esophageal bolus, and their erratic incidence following apparently adequate stimuli. It may be questioned, therefore, whether any real difference between these two forms of esophageal motility exists and whether, indeed, a swallowing center has any influence over the esophageal phase of primary peristalsis.

REFERENCES

1. Hwang, K. Mechanism of transportation of the content of the esophagus. J. appl. Physiol. 1954, **6**, 781.
2. Creamer, B., and Schlegel, J. Motor responses of the esophagus to distention. J. appl. Physiol. 1957, **10**, 498.
3. Meltzer, S. J. On the causes of the orderly progress of the peristaltic movements in the oesophagus. Amer. J. Physiol. 1899, **2**, 266.
4. Meltzer, S. J., and Auer, J. Vagus reflexes upon oesophagus and cardia. Brit. med. J. 1906, **2**, 1806.
5. Fleshler, B., Hendrix, T. R., Kramer, P., and Ingelfinger, F. J. Resistance and reflex function of the lower esophageal sphincter. J. appl. Physiol. 1958, **12**, 339.
6. Fyke, F. E., Jr., Code, C. F., and Schlegel, J. F. The gastroesophageal sphincter in healthy human beings. Gastroenterologia (Basel) 1956, **86**, 135.
7. Kramer, P., and Ingelfinger, F. J. I. Motility of the human esophagus in control subjects and in patients with esophageal disorders. Amer. J. Med. 1949, **7**, 168.
8. Kramer, P., and Ingelfinger, F. J. II. Cardiospasm, a generalized disorder of esophageal motility. Amer. J. Med. 1949, **7**, 174.
9. Ingelfinger, F. J., and Abbott, W. O. Intubation studies of the human small intestine. XX. The diagnostic significance of motor disturbances. Amer. J. dig. Dis. 1940, **7**, 468.
10. Butin, J. W., Olsen, A. M., Moersch, H. J., and Code, C. F. A study of esophageal pressures in normal persons and patients with cardiospasm. Gastroenterology 1953, **23**, 278.
11. Burget, G. E., and Zeller, W. E. A study of the cardia in unanesthetized dogs. Proc. Soc. exp. Biol. (N. Y.) 1936, **34**, 433.
12. Bosma, J. F. Deglutition: Pharyngeal stage. Physiol. Rev. 1957, **37**, 275.
13. Ingelfinger, F. J. Esophageal motility. Physiol. Rev. 1958, **38**, 533.
14. Andrew, B. L. The nervous control of the cervical oesophagus of the rat during swallowing. J. Physiol. 1956, **134**, 729.

THE EFFECT OF EXPERIMENTALLY INDUCED HYPERVOLEMIA ON CARDIAC FUNCTION IN NORMAL SUBJECTS AND PATIENTS WITH MITRAL STENOSIS *

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The physiologic differences between various types of laboratory preparations and unanesthetized man have been in part responsible for a wide diversion of opinion concerning the control of cardiac function in man (1). While numerous studies of the regulation of cardiac performance have been carried out in animals, relatively few studies have been done in man(2). The present work was undertaken in an attempt to clarify some of the mechanisms concerned with the control of cardiac function in man and has included studies in normal subjects and patients with mitral stenosis.

During a study of the relation existing between renal and cardiac function, rapid intravenous infusions were given to normal individuals and patients with various types of cardiac disorders (3). When the infusions consisted of isotonic solutions of dextran [1] it was possible to analyze the relation of right heart filling pressure to cardiac output or right ventricular work, in a manner which closely simulated that used by Sarnoff and Berglund in the anesthetized dog (4), or Warren, Brannon, Weens and Stead in man (5). The present report is concerned with the effects on cardiac performance of varied degrees of expansion of the blood volume during 32 studies in 31 normal subjects and 16 studies in 15 patients with mitral stenosis.

MATERIAL

Normal Subjects. Twenty-seven normal volunteers who were either policemen, medical students, or nurses were studied on an ambulatory basis, each resting for an hour before and after the study. On the basis of a history and physical examination none of these subjects showed evidence of cardiac or renal disease. In addition, four patients hospitalized because of cardiac symptoms but lacking objective findings of heart disease were studied. One of these subjects (No. 864) had high control values for oxygen consumption (339 and 358 ml. per minute), pulse rate (109 and 118 beats per minute) and cardiac index (7.06 and 6.68 ml. per minute per $M.^2$ body surface area [B.S.A.]). The oxygen consumption, pulse rate and cardiac index remained high throughout the study. This subject did not give a history suggestive of hyperthyroidism and the renal excretion and thyroid uptake of I^{131} as well as the protein bound iodine were within normal limits.

One subject (No. 948 and 976) was studied on two occasions three and one-half months apart.

The age of the subjects ranged between 16 and 49 years. Nineteen subjects were male and 12 were female.

Patients. Seventeen patients with rheumatic heart disease and mitral stenosis were studied. The data in two patients have been excluded from the results. One of these patients developed an allergic reaction to dextran manifested by urticaria and a marked fall in blood pressure, while the other patient was unable to tolerate the procedure due to the severity of her cardiac disease. All of the patients had the classic signs of mitral stenosis. Most of the patients were only moderately limited by their heart disease and were classified functionally in group I or II according to the criteria of the American Heart Association. Two of the patients (Nos. 873 and 990) were more severely affected and were classified in group III. One of the patients was studied both before and after commissurotomy (No. 994 and 1009). This patient and one other (No. 979) had atrial fibrillation. All other patients had sinus rhythm. None of the patients were receiving cardiac medications at the time of the study except for three patients (Nos. 873, 994 and 1009) who were fully digitalized. The patients ranged in age from 26 to 49 years. Eleven patients were female and four were male.

Figure 1 shows the age and heart size of the patients as well as the physiologic data prior to the start of the intravenous infusions. The patients receiving infusions of dextran in isotonic saline solution had somewhat larger hearts, slightly lower cardiac indices and higher pulmonary arterial pressures than those receiving infusions of dextran in 5 per cent glucose and water.

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[1] Macrodex Rx® of constant molecular size was generously supplied by Pharmacia Inc., Upsala, Sweden.

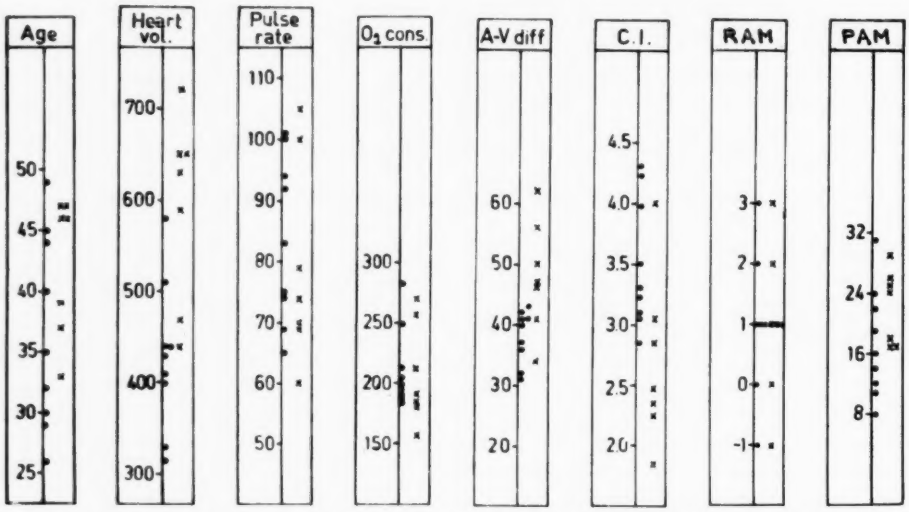

FIG. 1. CONTROL DATA OBTAINED ON ALL PATIENTS RECEIVING INFUSIONS OF DEXTRAN IN 5 PER CENT GLUCOSE AND WATER (BLACK DOTS) AND DEXTRAN IN ISOTONIC SALINE SOLUTION (X)

Heart volume is given in ml. according to Liljestrand's method. RAM designates right atrial mean pressure in mm. Hg; PAM, pulmonary arterial mean pressure in mm. Hg; PR, pulse rate per minute; O_2 cons., oxygen consumption in ml. per minute; CI, cardiac index in L. per minute per M.^{2} B.S.A.

METHODS

The pulmonary artery was catheterized according to the method of Cournand and Ranges (6–8). In four normal individuals (Nos. 855, 864, 907 and 926) and in three patients (Nos. 858, 863 and 925) a double lumen catheter was used. In two normal individuals (Nos. 867 and 986) and in five patients with mitral stenosis (Nos. 897, 967, 969, 979 and 994) two catheters were introduced, the tip of one catheter being placed in the pulmonary artery and the tip of the other in the right atrium. In all other studies the right atrial pressure was measured during passage of the catheter into the pulmonary artery, and again on withdrawal of the catheter just before the intravenous infusion was ended.

An indwelling arterial needle was placed in the brachial artery. Cardiac output was determined according to the direct Fick principle with simultaneous sampling of expired air and blood from the pulmonary and brachial arteries during a two minute period (9). The blood gases were determined in duplicate according to the technique of Van Slyke and Neill (10). Duplicate analyses of the expired air were carried out in a Haldane apparatus.

Blood samples for hemoglobin determinations were obtained periodically from the indwelling arterial needle. Hemoglobin content was measured from the amounts of oxyhemoglobin read in a Beckman spectrophotometer. The percentage decrease in hemoglobin was used as an indication of the increase in blood volume. Pressures were measured by Elema strain-gage manometers, the zero point of reference being 5 cm. below the sternum at the level of the fourth costochondral junction.

The right ventricular work was calculated using the following formulae: Right ventricular work index (RVWI) in Kg. per minute per M.^{2} B.S.A. = cardiac index × (mean pulmonary arterial pressure − mean right atrial pressure) × 13.6/1,000.

Right ventricular stroke work index (RVSWI) in Gm.-M. per beat per M.^{2} B.S.A. = stroke index × (mean pulmonary arterial pressure − mean right atrial pressure) × 13.6.

Procedure. All studies were performed in the morning with the subject recumbent and in the postabsorptive state. Since the study included an evaluation of renal function and renal hemodynamics the patient was initially given a priming dose of inulin and para-aminohippurate intravenously. During the remainder of the study these substances were given at a constant rate with a motor driven syringe. As part of the renal study, collections of urine were begun 30 minutes following the priming dose of inulin and para-aminohippurate and were repeated every 10 to 15 minutes during the remainder of the procedure. Blood samples for the clearance determinations were obtained from the brachial artery at the time of each collection of urine.

Approximately one hour after the priming doses of inulin and para-aminohippurate had been given and 30 minutes after the catheter or catheters had been positioned, peripheral and pulmonary arterial blood samples were obtained with the collection of expired air and the measurement of right heart and peripheral arterial pressures. In most individuals two control determinations of the cardiac output were obtained one to two minutes apart. The mean of these two values was used for com-

parison with the data obtained as the study progressed (9). Similar duplicate determinations of cardiac output were not made during the remainder of the study.

The infusion was begun immediately following the determination of the control values for oxygen consumption, arteriovenous oxygen difference, pulse rate and right heart and peripheral pressures.

Initial studies carried out with infusions of dextran alone proved unsatisfactory due to the occurrence of hemolysis. In two normal subjects (Nos. 851 and 855) and one patient (No. 858), a 10 per cent solution of dextran in water was used. In all subsequent studies dextran was infused as a 6 or 8 per cent solution with either 5 per cent glucose and water or isotonic saline solution. No evidence of hemolysis occurred with infusions of dextran solutions of this type. Thus, in 18 normal subjects and 15 patients with mitral stenosis, 6 per cent dextran was given either in 3 per cent glucose and water (13 normal subjects and nine patients with mitral stenosis) or in isotonic saline solution (five normal individuals and seven patients with mitral stenosis). In 12 normal subjects infusions of 5 per cent glucose and water (three subjects), isotonic saline solution (five subjects) and distilled water (four subjects) were given.

The infusions were given with a syringe driven by a motor at a constant rate so that approximately 25 ml. of fluid were injected per minute (11). Infusions of distilled water were given through a catheter, the tip of which lay in the right atrium. All other types of infusions were given through a polyethylene catheter which was inserted for a short distance into one of the antecubital veins. In normal subjects, solutions containing dextran were ordinarily given for 60 minutes. All other infusions in the normal individuals were given for periods of up to 100 minutes. In patients with mitral stenosis the infusions of dextran were terminated 30 to 50 minutes after their onset due to the rapid rise in pulmonary arterial pressure that ordinarily occurred at this time.

Cardiac output ordinarily was measured twice during the period of the infusion and again, in some instances, 15 to 20 minutes following the termination of the infusion. Measurement of right heart and peripheral pressures were carried out at 10 to 15 minute intervals at the onset of the infusion and more often during its later phases. All of the physiologic measurements were made without interrupting the infusion once it was begun.

No complications were noticed except in the two patients mentioned previously. None of the other subjects manifested signs of apprehension, dyspnea, orthopnea or pulmonary edema despite increases in blood volume of approximately 25 per cent and marked elevation of pulmonary arterial and right atrial pressures.

RESULTS

The necessity for infusing large amounts of water, glucose or saline with the dextran lead to a study of the cardiovascular effects of these fluids when they were given in a manner similar to that in which the dextran solutions were infused. Although infusions of distilled water produced hemolysis when given into a peripheral vein, no hemolysis developed when the infused water was given through a catheter, the tip of which lay in the right atrium.

In normal subjects infusions of isotonic saline solution, 3 per cent glucose in water and distilled water (Table I) produced a mean increase in blood volume of 5.3 per cent during the first 40 minutes of the infusion and 8.4 per cent when the infusion was continued for periods up to 80 minutes. Increases in blood volume of this magnitude produced no significant changes in heart rate, oxygen consumption, arteriovenous oxygen difference, cardiac index, stroke index, right atrial mean pressure and brachial arterial mean pressure (Table II). The mean value for pulmonary arterial mean pressure rose 2.5 mm. Hg or 27 per cent ($0.05 > p > 0.02$). Right ventricular stroke work index could be evaluated in only four studies rising less than 20 per cent in two subjects and 69 and 122 per cent in the other two individuals.

When dextran was given, with either 5 per cent glucose in water (Table III) or isotonic saline solution (Table IV), to normal subjects or patients without cardiovascular disease, the mean increase in blood volume was 12.9 per cent during the first 40 minutes of the infusion and 22.5 per cent when the infusion was continued for periods up to 60 minutes (Table V). A mean increase in blood volume of 12.9 per cent was not accompanied by significant changes in oxygen consumption, stroke index, brachial arterial mean pressure and the right ventricular stroke work index. Significant changes were found in the mean values for the arteriovenous oxygen difference (-4.6 ml. per L. or -12 per cent, $p < 0.001$), cardiac index ($+0.65$ L. per minute per M.^{2} B.S.A. or $+17$ per cent, $0.01 > p > 0.001$), right atrial mean pressure ($+4.1$ mm. Hg or $+168$ per cent, $p < 0.001$). The pulse rate increased four beats per minute or $+5$ per cent ($0.05 > p > 0.02$).

During the latter period of the infusion when the mean increase in blood volume was 22.5 per cent, brachial arterial mean pressure was not significantly changed over control values. All other physiologic measurements were altered

TABLE I

Normal subjects

Case no.	Sex	Age	Body surface area	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressure,† *mm. Hg* RA M	RV S	RV D	PA S	PA D	PA M	BA S	BA D	BA M	Right ventricular work index	Right ventricular stroke work index	Decrease in hemoglobin
			M.²	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./M.² B.S.A.*	*ml./beat/M.² B.S.A.*										*Kg. M./min./M.²*	*Gm. M./beat/M.² B.S.A.*	%
								A. 3 per cent glucose and water													
744	F	18	1.52	C	84	281	34	5.49	65	2			16	8	11	119	79	96	0.67	8.0	
				18	84	243	43	3.72	44				19	9	14	116	70	88			
				60	87	250	37	4.42	51				21	8	15	131	83	102			
745	M	22	2.01	C	64	353	40	4.37	68	2	18	2	15	5	9	104	62	75	0.42	6.5	
				7	65	427	29	7.30	112				16	6	10	107	65	81			
				98	66	412	37	5.62	85				18	7	12	117	70	87			
				112	76	505	33	7.68	101	1			15	5	9	113	65	83	0.84	11.0	
746	F	21	1.61	C	74	269	36	4.68	63	3			17	7	12	115	62	92	0.57	7.7	
				11	87	291	35	5.24	60				17	6	12	128	67	91			
				48	81	273	33	5.16	64				15	7	10	126	67	89			
				80	91	275	31	5.60	62				16	7	11	130	67	91			
								B. Distilled water													
882	M	30	2.06	C	98	349	40	4.29	44						11						
				35	95	378	35	5.24	55				25	9	12	130	67	83			4
				64	91	357	32	5.35	59				21	7	12	127	67	88			6
886	F	22	1.56	CI	77	199	31	4.10	53				15	5	8	100	54	69			
				CII	77	187	31	3.87	50												
				25	69	173	31	3.59	52				18	7	12	104	57	72			2
				50	80	186	31	3.81	48				18	7	11	104	58	73			4
895	M	26	1.83	CI	56	246	50	2.70	48	2									0.22	3.9	
				CII		199	44	2.46	44				13	5	8	98	59	69	0.20	3.6	
				31	56	234	52	2.48	44												5
				34		222	45	2.73	49				15	7	10	102	65	77			5
				58	53	209	49	2.33	44												6
				62		223	50	2.45	46	3			15	7	10	102	65	74	0.23	4.4	6
944	M	32	1.97	CI	66	248	38	3.31	50												
				CII	71	258	39	3.39	48				13	5	9	129	73	87			
				22	63	232	41	2.90	46				16	7	11	127	69	92			2
				53	71	236	39	3.05	43				16	6	10	120	64	84			6
								C. Physiologic saline solution													
916	M	28	2.06	CI	67	282	41	3.32	50	−1									0.36	5.4	
				CII	67	292	41	3.44	51				13	3	7	118	71	86	0.37	5.5	
				31	69	282	34	4.01	58				13	4	8	120	72	88			3
				56	66	296	39	3.73	57				16	6	10	128	79	89			5
				80	66	265	38	3.40	52	0	16	1	14	4	9	125	76	93	0.42	6.4	5
922	M	43	2.12	CI	63	293	49	2.84	45	3											
				CII	63	301	53	2.68	43	3						138	83	102			
				32.5	52	279	43	3.08	59	3						132	76	98			6
				68	62	295	40	3.44	55	2						136	80	92			8
1033	M	31	1.85	CI		203	30	3.64	44	1									0.30	3.6	
				CII	82	198	32	3.38	41				15	3	7	132	72	95	0.28	3.3	
				27	75	244	35	3.79	51				19	7	11	133	73	95			10
				44.5	71	243	34	3.84	54				20	7	12	129	66	95			14
1034	M	40	1.91	CI		237	38	3.30	54	3									0.31	5.1	
				CII	61	224	32	3.64	60				17	5	10	137	88	107	0.35	5.7	
				21	62	234	32	3.85	62				20	7	12	138	90	102			9
				41.5	67	278	35	4.16	62				23	9	15	142	90	111			11
				57.5	65	243	31	4.12	63	4	27	7	27	7	18			104	0.78	12.0	13
							BA–RA														
1036	F	28	1.74	CI		208	40	3.01	44												
				CII	68	195	36	3.09	45	3						132	71	88			
				22.5	69	269	34	4.52	66	6						138	73	90			7
				43.5	68	239	33	4.16	61	7						136	73	89			12
				59.5	70	266	38	4.07	58	6						133	68	87			14

* C designates control determination.

† Abbreviations are: RA, right atrium; RV, right ventricle; PA, pulmonary artery; BA, brachial artery; M, mean; S, systolic; D, diastolic.

TABLE II

Normal subjects: H_2O, physiologic saline solution and 3 per cent glucose in H_2O

			Difference from control values during infusion	
		Control	20–39 min.	39 min.
Heart rate (*beat/min.*)	N*	12	9	12
	M	71.9	−3.4	0
	SE	3.39	1.57	1.49
	SD	11.76	4.72	5.15
	p		0.1>p>0.05	0
Oxygen consumption (*ml./min.*)	N	12	9	12
	M	261.6	17.9	14.7
	SE	15.75	8.76	7.66
	SD	54.57	26.27	26.55
	p		0.1>p>0.05	0.1>p>0.05
Arteriovenous oxygen difference (*ml./L.*)	N	12	9	12
	M	38.6	−2.1	−1.9
	SE	1.72	1.43	1.28
	SD	5.96	4.29	4.42
	p		0.2>p>0.1	0.2>p>0.1
Cardiac index (*L./min./M.² B.S.A.*)	N	12	9	12
	M	3.74	0.36	0.35
	SE	0.245	0.204	0.197
	SD	0.847	0.613	0.684
	p		0.2>p>0.1	0.2>p>0.1
Stroke index (*ml./beat/M.² B.S.A.*)	N	12	9	12
	M	52.3	7.2	4.6
	SE	2.57	2.54	2.75
	SD	8.92	7.63	9.51
	p		0.05>p>0.02	0.2>p>0.1
Right auricular mean pressure (*mm. Hg*)	N			
	M	9		6
	SE	2.0		0.6
	SD	0.44		0.601
	p	1.32		1.5
				0.5>p>0.3
Pulmonary artery mean pressure (*mm. Hg*)	N	10	7	10
	M	9.2	2.3	2.5
	SE	0.55	0.47	0.95
	SD	1.75	1.25	2.99
	p		0.01>p>0.001	0.05>p>0.02
Brachial artery mean pressure (*mm. Hg*)	N	11	8	11
	M	87.8	1.4	1.7
	SE	3.79	1.53	1.74
	SD	12.58	4.34	5.76
	p		0.4>p>0.3	0.4>p>0.3
Right ventricular stroke work index (*Gm. M./beat/M.² B.S.A.*)	N	7		4
	M	5.8		1.6
	SE	0.66		
	SD	1.75		
	p			
Decrease in hemoglobin (%)	N		9	9
	M		5.3	8.4
	SE			
	SD			
	p			

* Abbreviations are as follows: N, number of observations; M, mean; SE, standard error; SD, standard deviation; p, p value.

TABLE III

Dextran with 5 per cent glucose in water

Case no.	Sex	Age	Body surface area	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressure,† *mm. Hg*									Right ventricular work index	Right ventricular stroke work index	Decrease in hemoglobin
										RA	RV		PA			BA					
										M	S	D	S	D	M	S	D	M			
			M.²	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./ M.² B.S.A.*	*ml./beat/ M.² B.S.A.*										*Kg. M./min./ M.² B.S.A.*	*Gm. M./beat/ M.² B.S.A.*	%
									A. Normal subjects												
851	M	19	1.93	CI	70	280	51	3.01	43										0.25	3.5	
				CII	70	267	46	2.99	43	5	21	6	17	6	11	103	73	90	0.24	3.5	
				17.5	62	274	47	3.02	49				20	10	15	110	69	86			8
				50.5	66	291	34	4.48	68	10			28	15	19	117	77	95	0.55	8.3	18
855	M	24	1.83	CI	74	234	32	4.01	54										0.33	4.4	
				CII	75	240	33	4.00	53		19	3	16	4	9	103	71	82	0.33	4.3	
				11.5	70	224	35	3.48	50		19	4	19	5	9	99	66	81	0.24	3.4	2
				22	69	262	35	4.10	59		19	5	21	6	10			79	0.28	4.0	5
				40.5	74	244	33	3.99	54		24	8	23	8	14	121	69	89	0.33	4.4	11
				10 aft.	79	262	38	3.77	49		25	7	25	8	13	116	67	88	0.32	4.0	13
867	F	21	1.63	CI	78	214	31	4.28	55										0.52	6.7	
				CII	80	189	33	3.52	44	0			15	5	9	113	58	80	0.43	5.4	
				23	75	221	19	7.10	95	7			22	11	15	111	61	77	0.77	10.3	18
				41	73	219	30	4.55	62	10			22	13	18	113	67	80	0.50	6.7	26
				58	80	224	31	4.45	56	12			26	16	22	110	62	77	0.61	7.6	31
894	F	25	1.45	CI	70	216	41	3.61	56										0.53	7.1	
				CII	70	225	40	3.85	55	3			19	7	13	112	67	76	0.52	7.5	
				23	75	204	33	4.32	58				25	12	18	105	64	77			16
				43.5	78	187	31	4.21	52				28	16	22	115	70	80			25
				53.5	83	193	31	4.30	52	11			28	16	25	107	64	77	0.82	9.9	28
926	M	42	2.04	CI	58	320	41	3.87	67												
				CII	55	283	38	3.70	67	1	25	5				97	67	84			
				19	59	294	36	4.00	68	3	29	1				112	68	86			13
				41	69	307	39	3.90	57	5	36	10				119	71	87			23
902	F	26	1.82	CI	71	227	34	3.68	52										0.35	5.0	
				CII	66	197	36	3.01	46	3			17	7	10	115	67	81	0.29	4.4	
				23.5	82	241	39	3.38	41				22	10	15	118	66	82			11
				50.5	79	244	29	4.62	58						19	128	74	87			18
				68	79	242	28	4.76	60	8			29	14	21	123	68	86	0.84	10.6	27
907	F	23	1.63	CI	85	220	29	4.62	54										0.50	5.9	
				CII	86	217	29	4.61	54		18	5	20	7	13	145	82	100	0.50	5.9	
				32.5	108	231	29	4.96	46		29	12	33	17	26	155	84	104	0.94	8.8	13
				48	111	230	20	7.23	65		32	13	33	19	27	150	83	101	1.38	12.4	15
				20 aft.	110	241	25	5.91	54		27	9	28	15	21	154	84	98	0.96	8.8	11

* C designates control determination; aft., time after infusion terminated.
† Abbreviations are: RA, right atrium; RV, right ventricle; PA, pulmonary artery; BA, brachial artery; M, mean; S, systolic; D, diastolic.

TABLE III—*Continued*

Case no.	Sex	Age	Body surface area	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressure,† *mm. Hg*									Right ventricular work index	Right ventricular stroke work index	Decrease in hemoglobin
										RA	RV		PA			BA					
										M	S	D	S	D	M	S	D	M			
			M.²	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./ M.² B.S.A.*	*ml./beat/ M.² B.S.A.*										*Kg. M./min./ M.² B.S.A.*	*Gm. M./beat/ M.² B.S.A.*	%
								A. Normal subjects													
948	M	34	1.78	CI	69	236	52	2.53			21	4									
				CII	68	254	53	2.69	40				12	4	8	132	86	105			
				28	68	285	44	3.66	54				19	7	12	139	87	101			10
				44	71	287	43	3.75	53	3	21	6	21	10	13	140	87	103	0.51	7.2	16
964	M	24	1.94	CI	77	290	31	4.86	63	0	13	3	14	8	11	113	59	76	0.73	9.4	
				CII	74	282	34	4.24	57				12	6	10	110	56	75	0.58	7.8	
				34	78	285	30	4.95	63				19	12	16	105	56	75			13
966	M	34	2.17	CI	63	289	42	3.21	51	3									0.26	4.2	
				CII	63	270	44	2.83	45				13	4	9	80	53	61	0.23	3.7	
				32	71	274	36	3.52	50				23	12	17	82	51	62			11
972	F	40	1.76	CI	81	221	34	3.69	46	1									0.30	3.8	
				CII	79	235	36	3.72	47				12	3	7	121	68	89	0.30	3.8	
				26.5	78	225	31	4.07	52				16	5	13	122	67	89			15
								B. Patients without cardiac disease													
899	F	31	1.79	CI	94	220	33	3.69	39	1	22	4							0.35	3.7	
				CII	94	209	29	4.01	43				13	4	8	103	59	70	0.38	4.1	
				40	93	248	30	4.62	50												15
				44	93	227	33	3.84	41				22	10	17						17
				61	88	241	31	4.33	49				25	14	18				0.82	9.3	23
				65	88	232	31	4.21	48	4			25	14	20	107	57	70	0.92	10.4	24
864	M	18	1.90	CI	109	358	27	7.06	65										0.77	7.1	
				CII	118	339	27	6.68	57	1	21	3			11	142	75	90	0.73	6.2	
				23	109	370	25	7.67	70		27	5	23	8	12	133	70	86	0.73	6.7	10
				40	107	350	21	8.90	83		25	5	26	8	14	136	73	91	1.09	10.2	16
896	F	16	1.56	CI	74	184	36	3.24	44	2									0.48	6.6	
				CII	75	198	37	3.46	46				20	8	13	104	55	75	0.52	6.9	
				32	79	200	30	4.33	55				31	16	23	115	66	81			22
				35	78	219	29	4.78	61				34	19	26	118	64	82			24
				47	83	199	30	4.33	52	9			36	21	28	113	73	94	1.12	13.4	27
903	F	49	1.68	CI	90	221	32	4.10	46	2									0.72	8.1	
				CII	88	200	32	3.71	42				26	6	15	140	74	109	0.66	7.4	
				18	91	208	31	4.04	44				31	10	20	135	70	98			14
				38	95	186	27	4.07	43				29	13	23	137	74	98			
				50	99	236	25	5.68	57	7			33	14	22	130	67	100	1.16	11.6	27

TABLE IV

Normal subjects: Dextran with physiologic saline solution

Case no.	Sex	Age	Body surface area	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressure,† *mm. Hg*							Right ventricular cardiac work index	Right ventricular stroke work index	Decrease in hemoglobin
										RA	PA			BA					
										M	S	D	M	S	D	M			
			M.²	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./ M.² B.S.A.*	*ml./beat/ M.² B.S.A.*								*Kg. M./min./ M.² B.S.A.*	*Gm. M./beat/ M.² B.S.A.*	*%*
976	M	35	1.79	CI	69	232	44	2.95	43	1							0.36	5.3	
				CII	69	219	45	2.69	39		17	5	10	121	75	90			
				28	78	235	41	3.20	41		27	11	17	126	74	90			12
				44	82	267	33	4.49	55		29	11	20	120	73	93			19
				57	94	290	34	4.79	51		29	14	21	115	64	85			24
986	M	27	2.14	CI	71	306	38	3.74	53	2	18	5	11	141	77	96	0.46	6.5	
				CII	71	325	40	3.78	53	1	19	4	11	141	77	94	0.51	7.2	
				22	79	313	32	4.58	58	4	26	10	18	142	77	100	0.87	11.0	7
				45	87	337	29	5.36	62	6	34	13	25	150	78	103	1.39	16.0	16
				21 aft.	90	343	27	5.92	66	4	30	11	20	146	76	99	1.29	14.4	18
							BA–RA												
996	M	45	2.22	CI	86	300	40	3.35	39	1									
				CII	84	304	39	3.50	42	0				157	99	116			
				31	86	361	30	5.44	63	5				157	92	120			13
				48	98	364	32	5.16	53	7				153	89	117			19
				18 aft.	99	366	32	5.22	53	4				143	85	107			20
1003	M	36	1.87	CI	68	240	54	2.37	35	3							0.19	2.9	
				CII	61	289	53	2.89	47		16	6	9	136	89	105	0.24	3.8	
				21	60	279	48	3.10	52		23	11	15	138	85	109			11
				40	57	301	45	3.58	63		27	13	19	137	81	110			25
				56	57	270	38	3.77	66	10	30	15	22	142	82	106	0.62	10.8	36
				20 aft.	61	260	38	3.65	60	7				145	87	105			34
							BA–RA												
1006	M	32	2.14	CI	68	272	31	4.14	61										
				CII	68	281	30	4.32	64	2				140	91	108			
				14	76	251	28	4.17	55	5				142	88	105			4
				31	79	263	32	3.89	49	7				143	88	107			6
				12 aft.	78	271	27	4.78	61	5				137	91	107			7

* C designates control determination; aft., time after infusion terminated.

† Abbreviations are: RA, right atrium; RV, right ventricle; PA, pulmonary artery; BA, brachial artery; M, mean; S, systolic; D, diastolic.

TABLE V

Normal subjects: Dextran in physiologic saline solution and dextran in 5 per cent glucose in H_2O

		Control	Difference from control values during infusion	
			20–39 min.	39 min.
Heart rate (*beat/min.*)	N*	20	17	16
	M	76.1	+4.0	+7.3
	SE	2.87	1.87	2.61
	SD	12.85	7.73	10.46
	p		0.05>p>0.02	0.02>p>0.01
Oxygen consumption (*ml./min.*)	N	20	17	16
	M	253.8	+7.1	+18.7
	SE	9.68	6.57	6.15
	SD	43.31	27.08	24.62
	p		0.3>p>0.2	0.01>p>0.001
Arteriovenous oxygen difference (*ml./L.*)	N	20	17	16
	M	38.0	−4.6	−7.0
	SE	1.73	1.10	1.38
	SD	7.73	4.54	5.54
	p		p<0.001	p<0.001
Cardiac index (*L./min./M.² B.S.A.*)	N	20	17	16
	M	3.77	+0.65	+1.17
	SE	0.207	0.212	0.192
	SD	0.925	0.875	0.769
	p		0.01>p>0.001	p<0.001
Stroke index (*ml./beat/M.² B.S.A.*)	N	20	17	16
	M	49.3	+6.6	+10.0
	SE	1.90	3.17	2.58
	SD	8.51	13.08	10.30
	p		0.1>p>0.05	0.01>p>0.001
Right atrial mean or right ventricular indiastolic pressure (*mm. Hg*)	N	20	7	14
	M	2.3	+4.1	+5.5
	SE	0.32	0.86	0.73
	SD	1.45	2.27	2.74
	p		0.01>p>0.001	p<0.001
Pulmonary artery mean pressure (*mm. Hg*)	N	17	15	14
	M	10.4	+6.3	+9.9
	SE	0.52	0.85	1.06
	SD	2.15	3.28	3.97
	p		p<0.001	p<0.001
Brachial artery mean pressure (*mm. Hg*)	N	20	17	16
	M	89.1	+0.4	+2.1
	SE	3.27	1.04	1.56
	SD	14.62	4.30	6.26
	p		0.8>p>0.7	0.2>p>0.1
Right ventricular stroke work index (*Gm. M./beat/M.² B.S.A.*)	N	16	5	10
	M	5.6	+2.0	+4.4
	SE	0.42	0.91	0.89
	SD	1.69	2.04	2.82
	p		0.1>p>0.05	p<0.001
Decrease in hemoglobin (%)	N		17	16
	M		+12.9	+22.5
	SE		1.30	1.65
	SD		5.35	6.61
	p		p<0.001	p<0.001

* Abbreviations are as follows: N, number of observations; M, mean; SE, standard error; SD, standard deviation; p, p value.

TABLE VI

Patients with mitral stenosis

Infused solution		Control			15–39 min. after the onset of infusions		
		Dextran in physiologic saline solution	Dextran in 5% glucose and water	Combined values	Dextran in physiologic saline solution	Dextran in 5% glucose and water	Combined values
Heart rate (*beat/min.*)	N*	7	9	16	7	9	16
	M	79.5	83.6	81.8	3.9	.6	1.72
	SE	6.32	4.52	3.66	2.224	2.16	1.31
	SD	16.75	13.57	14.63	5.9	6.51	6.25
	p				0.2 > p > 0.1	0.8 > p > 0.7	0.3 > p > 0.2
Oxygen consumption (*ml./min.*)	N	7	9	16	7	9	16
	M	208	211	209.8	16.4	2.6	8.7
	SE	18.6	10.96	8.4	10.12	6.77	6.295
	SD	45.58	32.87	33.6	26.82	20.21	25.18
	p				0.2 > p > 0.1	0.8 > p > 0.7	0.2 > p > 0.1
Arteriovenous oxygen difference (*ml./L.*)	N	7	9	16	7	9	16
	M	48	38.11	42.44	−2.86	−2.77	−2.81
	SE	3.497	1.453	2.094	2.26	.686	1.04
	SD	9.266	4.36	8.376	5.99	2.059	4.81
	p				0.3 > p > 0.2	0.01 > p > 0.001	0.02 > p > 0.01
Cardiac index (*L./min./M.² B.S.A.*)	N	7	9	16	7	9	16
	M	2.707	3.489	3.147	.443	.3844	.410
	SE	.256	.183	.1548	.1767	.142	.1118
	SD	.678	.549	.619	.468	.427	.4475
	p				0.05 > p > 0.02	0.05 > p > 0.02	0.01 > p > 0.001
Stroke index (*ml./beat/M.² B.S.A.*)	N	7	9	16	7	9	16
	M	34.86	42.11	38.94	2.71	3	2.875
	SE	2.99	1.148	1.68	1.491	1.365	.976
	SD	7.91	3.446	6.72	3.949	4.096	3.903
	p				0.2 > p > 0.1	0.1 > p > 0.05	0.02 > p > 0.01
Right atrial mean or right ventricular indiastolic pressure (*mm. Hg*)	N	7	9	16	6	7	13
	M	1	2	1.56	3.83	4.7	4.31
	SE	.184	.842	.5	.749	1.267	.745
	SD	.4873	2.528	2.00	1.834	3.356	2.69
	p				0.01 > p > 0.001	0.01 > p > 0.001	p < 0.001
Pulmonary artery mean pressure (*mm. Hg*)	N	7	9	16	7	9	16
	M	22.29	17.44	19.56	18.285	9.888	13.56
	SE	1.73	2.43	1.658	1.267	1.173	1.658
	SD	4.59	7.29	6.634	3.355	3.519	5.442
	p				p < 0.001	p < 0.001	p < 0.001
Brachial artery mean pressure (*mm. Hg*)	N	7	9	16	7	9	16
	M	100.5	75.33	86.37	7.71	1.89	4.437
	SE	6.179	3.1175	4.75	2.356	2.419	1.821
	SD	16.368	12.49	18.91	6.242	7.258	7.284
	p				0.02 > p > 0.01	0.5 > p > 0.3	0.05 > p > 0.02
Right ventricular stroke work index (*Gm. M./beat/M.² B.S.A.*)	N	7	9	16	6	7	13
	M	9.73	8.9	9.26	7.45	4.086	5.638
	SE	.6995	1.624	.960	.818	.7837	.770
	SD	1.853	4.872	3.841	2.005	2.076	2.778
	p				p < 0.001	0.01 > p > 0.001	p < 0.001
Decrease in hemoglobin (%)	N				7	8	15
	M				14.57	18	16.28
	SE				1.97	1.32	1.207
	SD				5.219	3.742	4.671
	p				p < 0.001	p < 0.001	p < 0.001

* Abbreviations are as follows: N, number of observations; M, mean; SE, standard error; SD, standard deviation; p, p value.

significantly over the control values; pulse rate (+ 7.3 beats per minute or + 9.6 per cent, $0.02 > p > 0.01$), oxygen consumption (+ 18.7 ml. per minute or + 7 per cent, $0.01 > p > 0.001$), arteriovenous oxygen difference (− 7 ml. per L. or − 18 per cent, $p < 0.001$), cardiac index (+ 1.17 L. per minute per M.² B.S.A. or + 31 per cent, $p < 0.001$), stroke index (+ 10 ml. per beat per M.² B.S.A. or + 20 per cent, $0.01 > p > 0.001$), right atrial mean pressure (+ 5.5 mm. Hg or + 242 per cent, $p < 0.001$), and right ventricular stroke work index (+ 4.4 Gm.M per beat per M.² B.S.A. or + 78.6 per cent, $p < 0.001$).

During the control period, patients with mitral stenosis (Table VI) differed from normal subjects in having higher pulmonary arterial mean pressures (normals, 10.4 mm. Hg; patients, 19.5 mm. Hg) and greater right ventricular stroke work indices (normals, 5.6 Gm.M. per beat per M.² B.S.A.; patients, 9.26 Gm.M. per beat per M.² B.S.A.).

Although patients with mitral stenosis receiving infusions of dextran in 5 per cent glucose and

TABLE VII

Patients with mitral stenosis

Case no.	Sex	Age	Body surface area	Heart volume	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressures,† *mm. Hg*									Right ventricular stroke work index	Decrease in hemoglobin
											PCV	RA	RV		PA			BA			
											M	M	S	D	S	D	M	S	D	M	
			M.²	*ml./M.² B.S.A.*	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./ M.² B.S.A.*	*ml./beat/ M.² B.S.A.*										*Gm. M./beat/ M.² B.S.A.*	*%*
A. Dextran with 5 per cent glucose and water																					
858	F	30	1.45	315	C	94	184	36	3.52	37			20	4	18	7	12			72	4.0	
					23	83	216	37	4.01	48			33	11	32	15	22			87	7.2	12
					39	91	223	34	4.47	49			36	14	38	20	27			90	8.7	22
					12.5 aft.	114	256	30	5.86	51			40	11	35	20	27			85	11.1	29
863	F	44	1.53	510	C	83	204	41	3.25	39			29	7	30	10	16	89	43	59	4.8	
					17.5	88	203	39	3.52	40			36	11			22	90	49	58	6.0	10
					35.5	93	208	35	3.88	42			42	15	43	17	27	96	52	63	6.9	19
					7 aft.	94	215	37	3.80	40			40	13	44	18	27	92	49	62	7.6	21
873	M	35	1.77	440	C	101	282	37	4.31	43	7	−1			43	22	31	106	60	75	18.7	
					20	97	276	44	3.55	37					59	30	35	103	59	70		13
					33	99	294	36	4.62	47		2			61	31	42	103	56	72	25.6	20
897	F	29	1.65	400	C	69	212	40	3.21	47		0			17	4	8	107	57	74	5.1	
					20.5	70	218	33	4.05	58		2			18	7	11	99	54	69	7.1	10
					25	65	217	40	3.30	51		2			18	7	11	97	54	70	6.2	13
					46.5	65	239	37	3.89	60		7			25	11	16	107	60	72	7.3	19
					2 aft.	78	222	35	3.85	49					20	8	14	100	55	73		20
925	F	32	1.42	330	C	100	190	32	4.18	42	5	1	26	1	20	5	11	111	59	88	5.7	
					19	91	191	32	4.23	46		2	35	6	31	13	20	123	68	81	11.3	
					33.5	94	167	27	4.36	46		4	38	8	34	16	23	115	64	82	11.9	
958	M	49	1.89	440	C	74	248	42	3.12	42	11	1			22	8	14	141	75	98	7.4	
					32	79	237	41	3.09	39					34	17	23	135	71	101		22
					44	82	279	34	4.37	53		6			38	20	26	156	78	101	14.4	26
960	F	40	1.60	430	C	92	195	31	3.93	43	10	2			28	13	19	106	56	73	9.9	
					27	85	169	29	3.68	43					36	19	25	105	56	72		12
					48	107	173	34	3.17	30	24	7			44	26	35	106	56	75	11.4	20
967	F	45	1.50	580	C	65	187	41	3.04	47	21	3			38	16	24	106	58	80	13.4	
					25	74	188	38	3.33	45		8			47	21	33	109	59	80	15.3	18
					38	78	197	35	3.74	48		9			48	23	35	108	58	79	17.0	24
969	F	26	1.62	410	C	75	198	43	2.84	39		1			27	15	22	105	45	59	11.1	
					29	79	212	41	3.23	41		6			39	25	33	111	51	66	15.1	18
					47	89	243	40	3.72	42		7			40	29	34				15.4	20

* C designates control determination; aft., time after infusion terminated.
† Abbreviations are: RA, right atrium; RV, right ventricle; PA, pulmonary artery; BA, brachial artery; M, mean; S, systolic; D, diastolic; PCV, pulmonary capillary venous pressure.

TABLE VII—*Continued*

Case no.	Sex	Age	Body surface area	Heart volume	Time*	Heart rate	O_2 consumption	A-V O_2 difference	Cardiac index	Stroke index	Blood pressures,† *mm. Hg*										Right ventricular stroke work index	Decrease in hemoglobin
											PCV	RA	RV		PA			BA				
											M	M	S	D	S	D	M	S	D	M		
			M.²	*ml./M.² B.S.A.*	*min.*	*beat/min.*	*ml./min.*	*ml./L.*	*L./min./M.² B.S.A.*	*ml./beat/M.² B.S.A.*											*Gm. M./beat/M.² B.S.A.*	*%*
									B. Dextran in isotonic saline solution													
979	F	39	1.36	590	C	69	191	56	2.51	36	22	1	40	3	42	16	26	110	54	75	12.2	
					21	72	187	58	2.38	33		6			61	25	40	118	61	80	15.3	13
					31	77	203	58	2.59	34		7			64	26	43	117	61	81	16.6	15
981	F	37	1.58	440	C	100	213	34	3.96	40	12	−1			30	10	17	143	76	99	9.8	
					24	110	234	29	5.07	46					43	22	32	134	74	92		18
					37	111	231	29	5.09	46					49	27	38	136	74	96		24
990	F	46	1.69	630	C	105	157	50	1.86	18	11	0			42	20	29	173	101	130	7.1	
					19	111	229	47	2.88	26		5			73	31	50	173	97	134	15.9	10
991	M	33	1.47	470	C	79	185	41	3.07	39	21	3			40	18	25	122	75	95	11.7	
					19	75	206	48	2.90	39		4	57	12	57	27	41	129	75	103	19.6	10
994	M	47	1.95	650	C	60	271	62	2.24	37	10	2			32	12	18	146	83	98	8.1	
					18.5	52	256	60	2.18	42		5			40	15	24	151	87	105	10.9	10
					35.5	61	268	52	2.63	43		7			51	20	32	164	96	113	14.6	15
					aft. 19	53	257	51	2.57	48		6			47	18	31	154	88	112	16.3	20
1004	F	46	1.62	720	C	74	183	46	2.46	33	18	1			34	18	24	143	84	107	10.3	
					18	86	184	39	2.91	34					55	29	39	153	88	114		10
					29	88	186	37	3.09	35		4	66	8	64	36	47	162	95	120	20.5	18
					aft. 21	86	181	37	3.03	35		4						163	93	123		20
1009	M	47	1.92	650	C	70	257	47	2.85	41	10	1			32	11	17	146	86	101	8.9	
					17	73	251	49	2.69	37					49	18	27	157	91	107		5
					33	71	245	45	2.83	40		4	50	7	53	20	33	165	95	111	15.8	10
					aft. 19	77	269	45	3.09	40		3						154	86	109		12

water (Table VII) showed only slight differences from the normal group in their mean control values for cardiac index, stroke index and arteriovenous oxygen difference, patients infused with dextran in isotonic saline solution (Table VII) had significantly lower cardiac and stroke indices and higher arteriovenous oxygen differences than the normal volunteers.

The results of infusions of dextran for periods of 40 minutes in patients with mitral stenosis and normal subjects were similar in producing slight but not significant changes in heart rate, oxygen consumption and brachial arterial pressure, while blood volume, cardiac and stroke indices and right heart filling pressure were increased by roughly comparable amounts.

The results differed in the greater increase in mean pulmonary arterial pressure (normals, + 6.3 mm. Hg; patients, + 13.6 mm. Hg) (Figure 2), and right ventricular stroke work index (normals, + 2 Gm.M. per beat per M.² B.S.A.; patients, + 5.6 Gm.M. per beat per M.² B.S.A.) found in the patients. The difference was most marked in patients receiving infusions of dextran in isotonic saline solution in whom the mean increases in pulmonary arterial mean pressure and right ventricular stroke work index were 18 mm. Hg and 7.45 Gm.M. per beat per M.² B.S.A., respectively, approximately three times that found in normal subjects.

While mean increases in right heart filling pressures of 5.5 mm. Hg in normal subjects and 4.3 mm. Hg in patients were associated with significant increases in the mean values for cardiac and stroke indices and right ventricular stroke work index, the coefficients of the regressions of right heart filling pressures to the change in these indices were not significant (Figures 3–5).

Examination of the data in individual studies shows instances in which significant changes in right heart filling pressures were associated with changes in cardiac output which could not be considered significant, as the standard error of a single determination of cardiac output in this laboratory has been demonstrated to be 7.2 per cent (9). A 10 per cent variability in duplicate determinations of cardiac output has been shown elsewhere (12, 13).

In seven normal volunteers cardiac indices were altered by less than 15 per cent while right heart filling pressures rose 4 to 12 mm. Hg. Stroke indices were unaltered or fell in five subjects in association with increases in right filling pressures of 5 to 10 mm. Hg.

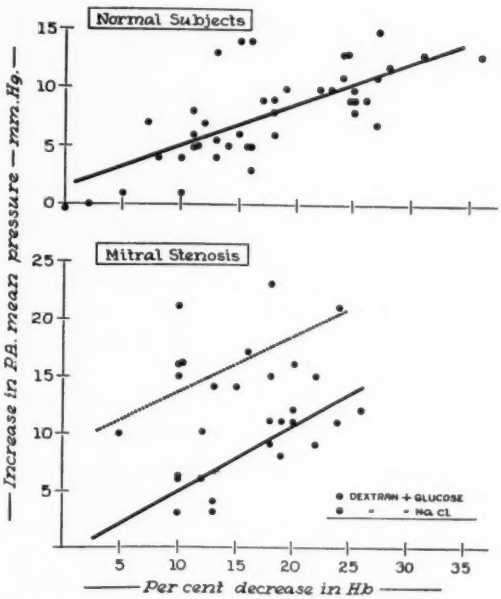

FIG. 2. REGRESSIONS FOR THE INCREASE IN PULMONARY ARTERIAL MEAN PRESSURE ON THE PERCENTAGE DECREASE IN HEMOGLOBIN

On the lower half of the figure separate regression lines are plotted for infusions of dextran in isotonic saline solution (dotted line) and dextran in 5 per cent glucose and water (solid line). The coefficients of the regression equations, $E = 0.357x + 1.653$ (normal subjects) and $E = 0.576x - 0.855$ (patients receiving infusions of dextran in 5 per cent glucose and water), are significant ($p < 0.001$ in each instance). The coefficient of the equation $E = 0.485x + 9.035$ in patients infused with dextran in isotonic saline solution is not significant ($0.2 > p > 0.1$).

In six patients cardiac indices showed changes of less than 10 per cent while right heart filling pressures rose 2 to 6 mm. Hg. Stroke indices were unaltered or increased 1 to 2 ml. in three patients with increases in right heart filling pressures of 1 to 6 mm. Hg, while in three other patients stroke indices fell 1 to 10 ml. in association with increases in right heart filling pressures of 1 to 6 mm. Hg.

In all patients and in all normal subjects, save one, the right ventricular stroke work index rose in association with increases in right heart

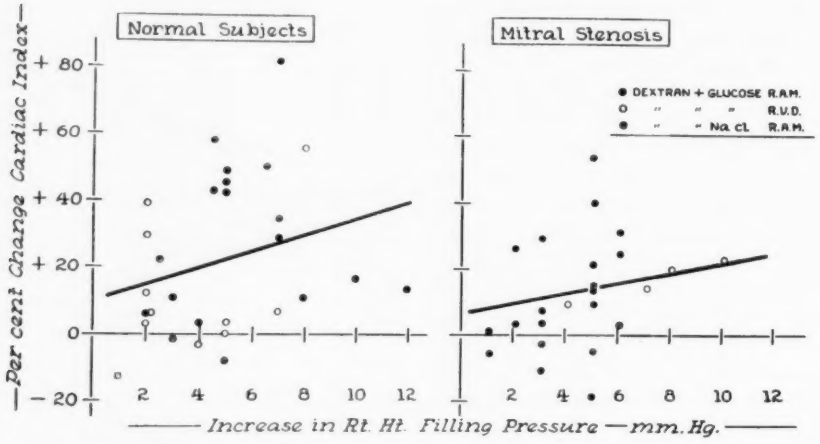

FIG. 3. REGRESSIONS FOR THE PERCENTAGE CHANGE IN CARDIAC INDEX ON THE INCREASE IN RIGHT HEART FILLING PRESSURE

RAM designates right atrial mean pressure; RVD, right ventricular end-diastolic pressure. The coefficients of the regression equations, $E = 2.53x + 9.974$ (normal subjects) and $E = 1.182x + 6.406$ (patients with mitral stenosis), are not significant ($p > 0.1$ and $p > 0.3$, respectively).

filling pressures (Figure 6) and elevation of blood volume (Figure 7). The rise in right ventricular stroke work index was sometimes small in relation to the increase in right heart filling pressures being less than 40 per cent in five patients and two normal subjects with increases in right filling pressures of 2 to 8 mm. Hg and 8 to 12 mm. Hg, respectively.

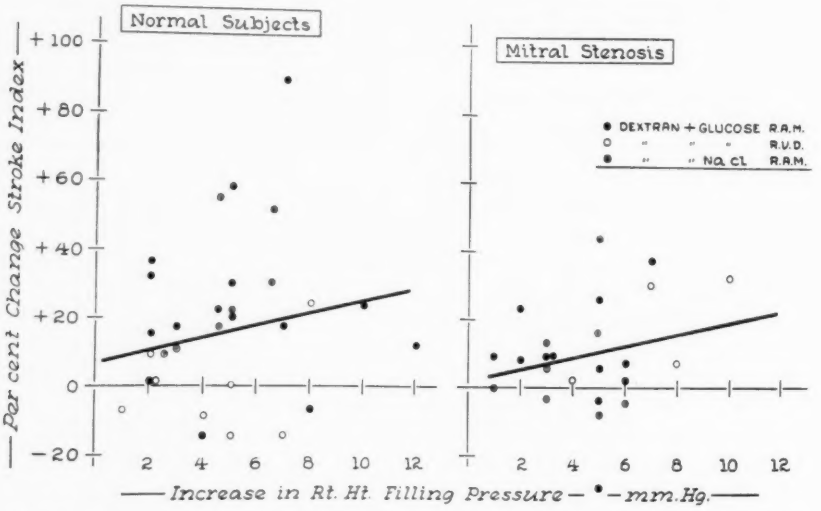

FIG. 4. REGRESSIONS FOR THE PERCENTAGE CHANGE IN STROKE INDEX ON THE INCREASE IN RIGHT HEART FILLING PRESSURES

RAM designates right atrial mean pressure; RVD, right ventricular end-diastolic pressure. The coefficients of the regression equations, $E = 1.76x + 6.896$ (normal subjects) and $E = 1.66x + 2.095$ (patients with mitral stenosis), are not significant ($p > 0.3$ and $p > 0.1$, respectively).

A significant linear relation was found between the percentage decrease in hemoglobin in normal subjects and the percentage change in cardiac index ($0.02 > p > 0.01$) (Figure 8), the percentage change in stroke index ($0.005 > p > 0.001$) (Figure 9), and the change in right ventricular stroke work index ($0.05 > p > 0.02$) (Figure 10). The correlation coefficients of 0.41, 0.36 and 0.47 relating the percentage decrease in hemoglobin in the normal subjects to these indices were also significant. In patients with mitral stenosis a significant linear relation was found between the percentage decrease in hemoglobin and the percentage change in cardiac index ($0.05 > p > 0.02$). The correlation coefficient of 0.37 was also significant. No significant linear relation was found between the percentage decrease in hemoglobin in the patients with mitral stenosis and the percentage change in stroke index or the change in right ventricular stroke work index.

DISCUSSION

The effect of rapid infusions of albumin (5), dextran (14) or saline (15, 16) on the cardiac output and pressures in the right atrium and pulmonary artery has been studied previously in man. While it has been found consistently that right atrial pressure rises as blood volume increases, the effect on cardiac output has varied, cardiac output remaining unchanged in one study (5) and rising in the others (14–16). This divergence in results may be due to variations in the magnitude of the blood volume increase, to physiologic differences among the subjects or to differences in the experimental procedures. In an effort to limit the effect of these variables, comparable studies were first carried out in normal volunteers and subsequently in patients with mitral stenosis.

In normal subjects infusions of 3 per cent glucose and water or isotonic saline solution increased blood volume 5 to 8 per cent with no significant change in right heart pressures and stroke or cardiac indices. Infusions of dextran in 5 per cent glucose and water or isotonic saline solution given in a similar manner to normal sub-

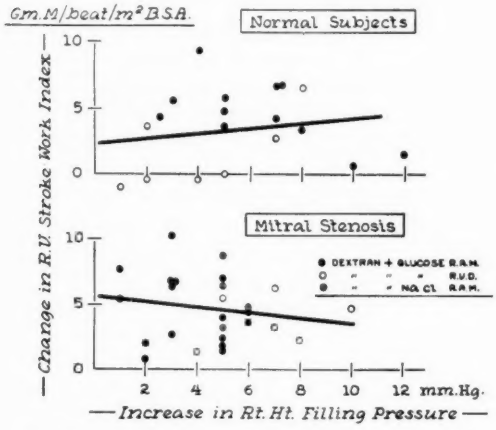

FIG. 5. REGRESSIONS FOR THE CHANGE IN RIGHT VENTRICULAR (RV) STROKE WORK INDEX ON THE INCREASE IN RIGHT HEART FILLING PRESSURE

RAM, right atrial mean pressure; RVD, right ventricular end-diastolic pressure. The coefficients of the regression equations, $E = 0.1754x + 2.46$ (normal subjects) and $E = 5.403 - 0.174x$ are not significant ($p > 0.3$ in each instance).

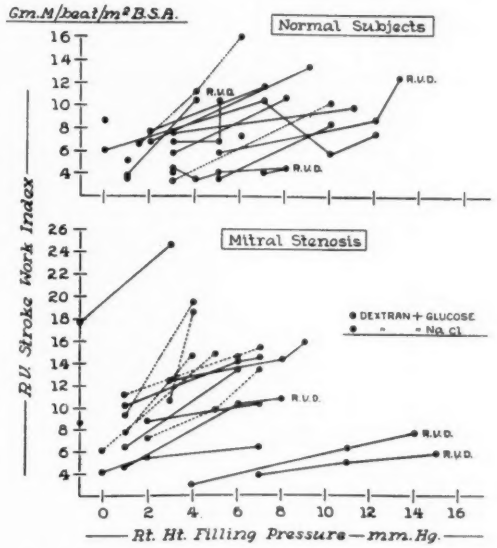

FIG. 6. THE RELATION BETWEEN RIGHT HEART FILLING PRESSURE AND RIGHT VENTRICULAR STROKE WORK INDEX IN EACH SUBJECT

The initial plots on the left hand side of the figure represent control values. All other plots occurred during the period of the infusions. Right atrial mean pressure was considered representative of right heart filling pressure except in those instances marked RVD, when right ventricular end-diastolic pressure was used.

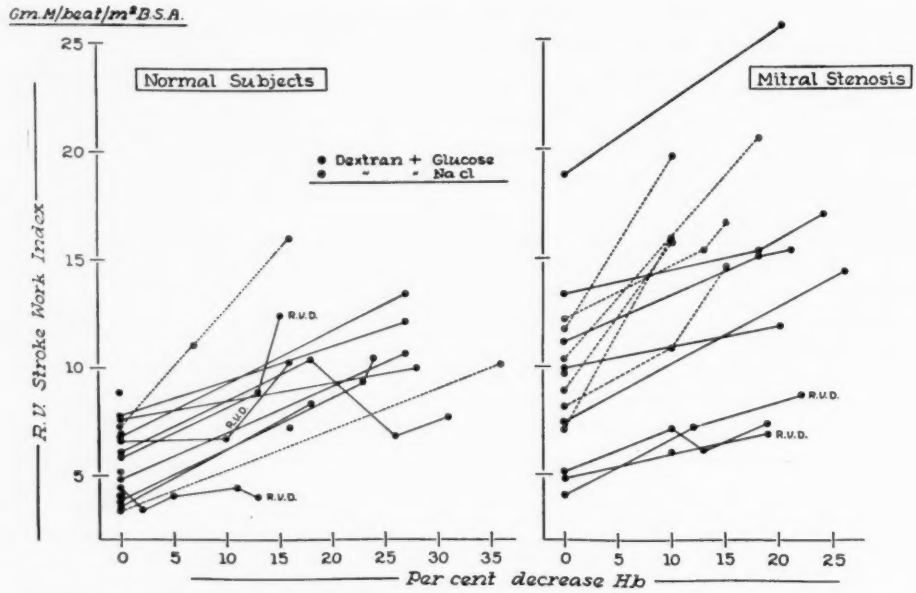

FIG. 7. THE RELATION BETWEEN THE PERCENTAGE DECREASE IN HEMOGLOBIN AND RIGHT VENTRICULAR STROKE WORK INDEX IN EACH SUBJECT

The plots on the left of the graph are control values; subsequent plots were obtained during the period of the infusion. Right atrial mean pressure was used in calculating right ventricular stroke work index except in those instances marked RVD, when right ventricular end-diastolic pressure was used.

jects and patients with mitral stenosis increased blood volume 22 and 16 per cent, respectively, and were associated with significant increases in right heart and pulmonary arterial pressures, the increase in pressures being related to the degree of blood volume expansion, a relation that had been found previously in anesthetized dogs (17, 18).

Right heart filling pressure increased to a mean value of 6 to 7 mm. Hg, a level commonly found in patients with right heart failure, yet the absence of a fall in cardiac output either in the normal subjects or the patients with mitral stenosis suggests that the heart was not in a failing state.

The rise in pulmonary arterial pressure was significantly greater in patients with mitral stenosis, a finding which might be due either to alterations known to occur in the pulmonary vascular bed of these patients or perhaps to the presence of right ventricular hypertrophy.

In these studies the correct evaluation of the effects of alterations in right heart filling pressures on cardiac output and cardiac work is dependent on the relation existing between measured and effective pressure as well as the significance of right atrial mean pressure as an indicator of the filling pressure in the right ventricle.

Changes in intrathoracic and intrapericardial pressures were not measured during these studies making it impossible to determine the effective right heart pressures. Although the change in effective right atrial pressure was probably less than the measured pressure change, the difference was felt to be no greater than 1 to 2 mm. Hg.

In those instances in which right atrial mean and right ventricular end-diastolic pressures were measured simultaneously the latter was 1 to 4 mm. Hg higher than the former during the control period. During the period of the infusion, right atrial mean pressure and right ventricular end-diastolic pressures rose in a comparable manner (Figure 11) as had been noted previously during exercise studies in patients with mitral stenosis (19).

Besides increasing blood volume and elevating right heart filling pressure, an infusion of dextran causes other physiologic changes which in themselves may play some role in the regulation of cardiac output. Increased cardiac outputs found in dogs during dextran infusions were shown to be the result of a marked anemia and the associated decrease in the oxygen carrying capacity of the blood (18, 20). In the present studies the magnitude of the anemia was less than in the animal studies cited above, hematocrits being altered less than 30 per cent and hemoglobin levels never falling below 10 Gm., a level which has not been found to be associated with elevation of cardiac output in studies of patients with chronic anemia (21).

Cardiac output and right heart filling pressures may be altered by the occurrence of tricuspid insufficiency during dextran infusions in animals with open pericardiums (22). Examinations of the atrial pressure curves recorded at the end of the infusion periods failed to reveal evidence of tricuspid insufficiency in either normal volunteers or patients with mitral stenosis.

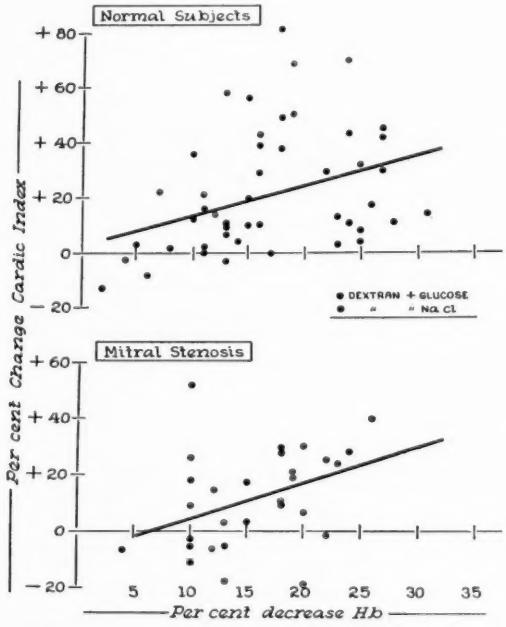

FIG. 8. REGRESSIONS FOR THE PERCENTAGE CHANGE IN CARDIAC INDEX ON THE PERCENTAGE DECREASE IN HEMOGLOBIN

The coefficient of the regression equations, $E = 1.058x + 4.18$ (normal subjects) and $E = 1.188x - 6.78$ (patients with mitral stenosis), are significant ($0.02 > p > 0.01$ and $0.05 > p > 0.02$, respectively).

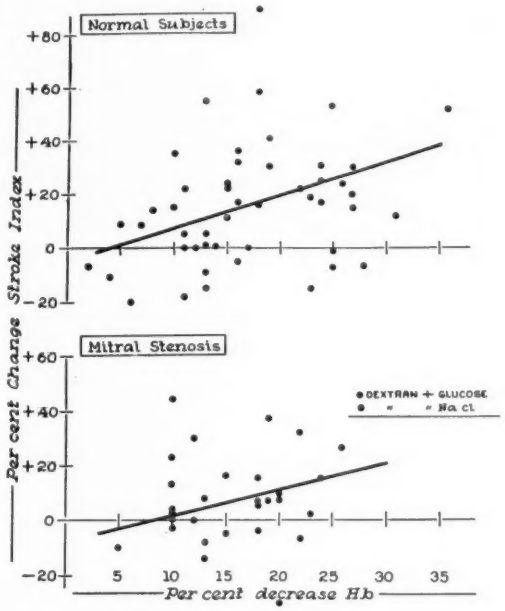

FIG. 9. REGRESSIONS FOR THE PERCENTAGE CHANGE IN STROKE INDEX ON THE PERCENTAGE DECREASE IN HEMOGLOBIN

The coefficient of the regression equation, $E = 1.2x - 4.95$ in normal subjects, is significant ($p < 0.005$). The coefficient of the regression equation, $E = 0.9133x - 6.78$ in patients with mitral stenosis, is not significant ($p > 0.1$).

Data on renal blood flow obtained during the present studies show that infusions of dextran are often associated with changes in renal hemodynamics. In normal subjects renal blood flow was not significantly altered during infusions of isotonic saline solution or 5 per cent glucose and water. When normal volunteers received infusions of dextran with 5 per cent glucose and water or isotonic saline solution, renal blood flow increased, in some instances, to twice the control values. Increases in renal blood flow of this magnitude, although partially explicable on the basis of the measured change in cardiac output, would appear also to be indicative of vasodilitation in the renal vascular bed.

While the data in normal subjects and patients

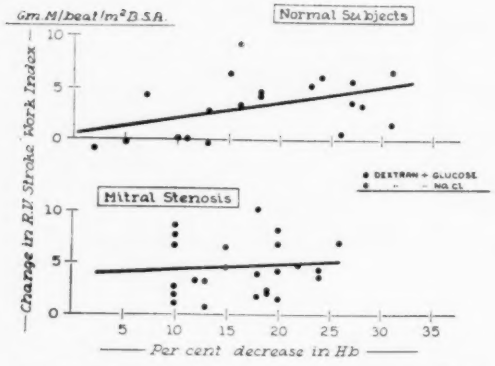

FIG. 10. REGRESSIONS FOR THE CHANGE IN RIGHT VENTRICULAR STROKE WORK INDEX ON THE PERCENTAGE DECREASE IN HEMOGLOBIN

The coefficient of the regression equation, $E = 0.15x + 0.656$ in normal subjects, is significant ($0.05 > p > 0.02$). The coefficient of the regression equation, $E = 0.044x + 3.829$ in patients with mitral stenosis, is not significant ($p > 0.9$).

with mitral stenosis show a mean rise in cardiac and stroke output occurring in association with increases in right heart filling pressures, no uniform way of relating these variables was found; indeed in 13 instances, changes in cardiac output of questionable significance occurred in the presence of increases in right heart filling pressures of 4 to 12 mm. Hg. Previous studies in subjects with induced fluid retention have in some instances also failed to show a relation between right heart filling pressure and cardiac output (23).

Increases in right heart filling pressures of the magnitude produced in these studies are not usually encountered in the intact normal individual even under conditions of moderate to severe exertion (24) when the cardiac output is elevated to a far greater extent than was encountered in this work (25). The occurrence of only slight to moderate increases in cardiac output in association with abnormal increases in right heart filling pressures in healthy subjects implies the importance of other mechanisms by which cardiac output may be elevated from normal levels to the higher values associated with the physiologic stresses of daily life.

The significance of increases in right heart filling pressure to the elevation of cardiac output in patients with mitral stenosis cannot be clearly assessed since it is well known that these patients may be unable to increase their cardiac outputs to the extent found in normal subjects. It appears, however, that the effect on cardiac output of increases in right heart filling pressures, within the range studied, was essentially the same in healthy subjects and in patients with mitral stenosis.

Changes in blood volume were also associated with significant increases in the mean values of cardiac and stroke indices in both patients and normal subjects. The relation between blood volume changes and these indices was significant in normal subjects. In patients with mitral stenosis the relationship was significant only as regards the cardiac index. There were again eight normal subjects and five patients in whom maximum increases in blood volume of 10 to 31 per cent were associated with changes in cardiac output of less than 15 per cent, a change which should be considered of questionable significance in view of the limitations of the methods utilized.

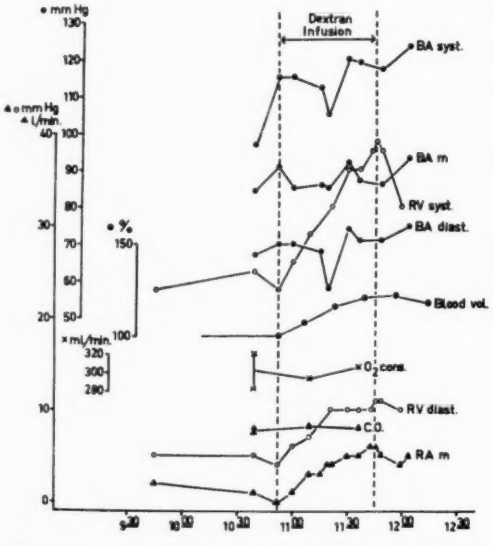

FIG. 11. DATA OBTAINED DURING AN INFUSION OF DEXTRAN IN 5 PER CENT GLUCOSE AND WATER IN A NORMAL SUBJECT (No. 926)

The vertical dotted lines represent the start and the finish of the dextran infusion. Syst. designates systolic; Diast., diastolic; M, mean.

The belief that right heart filling pressure is of dominant importance in the control of cardiac output in man has been based partly on studies conducted during pressure breathing (26), acute hemorrhage (27) and tilting (28, 29), experimental procedures which lead to a decreased venous return and a fall in the effective filling pressure of the right heart. Under these circumstances an ultimate fall in cardiac output would appear inevitable for the heart cannot pump out more blood than it receives. It does not necessarily follow that an increased venous return and an elevated cardiac filling pressure need be associated with an increased cardiac output.

The data in the present study suggest that the alterations in cardiac filling pressure and venous return cannot adequately explain the wide variations in cardiac output occurring in normal man; indeed the lack of a significant change in cardiac output in 13 subjects despite a steady increase in blood volume and right heart filling pressure suggests the presence of some mechanism by which cardiac output is maintained at a relatively constant level under these conditions.

In each subject, save one, increase in blood volume and elevation of right heart filling pressure were associated ultimately with an increase in right ventricular stroke work, a finding which is in accordance with Starling's law of the heart (30). However, no significant linear relation between these two variables and work was found except in the normal subjects where a significant linear relation existed between the percentage decrease in hemoglobin and the increase in right ventricular stroke work index. In individual subjects the increase in work for a given change in pressure was often far less than has been reported previously in animals. While this difference in the pressure volume relation (the ventricular function curve) of man and animal might be explained on the basis of the species studied, it might also be the result of the altered physiologic status of a laboratory preparation as compared to intact unanesthetized man. In a laboratory preparation, the normal state is sacrificed in varying degrees to reduce the number of variables under study to a minimum. In intact man these many variables operate simultaneously, each altering to some degree the manner in which the others would function were they to be operating alone.

Other investigators (2, 4) have felt that the response of the heart to a given filling pressure may vary widely as a result of changes presumably in myocardial tone or contractility; functions of cardiac muscle which appear at least in part to be under neurohumoral control. The presence of these neurohumoral factors in intact man and their absence in many laboratory preparations would appear to be the most likely explanation of the disparity between the magnitude of the work increase found in this study and that found in the animal studies cited above.

The greatest increase in work for a given increase in blood volume or filling pressure was found in those patients with mitral stenosis whose hemodynamic findings showed the most marked deviations from normal. Whether this difference between patients and normal subjects was due to the presence of right ventricular hypertrophy, or the result of a change in myocardial contractility or tone initiated by some neurohumoral mechanism, cannot be determined from the present data.

The present study is an attempt to place in proper perspective the relation of increased blood volume and filling pressure to cardiac function in intact man. The data in no way deny to cardiac muscle its inherent ability of responding to increased tension with increased force (Starling's law of the heart), all other variables remaining constant. The data do, however, show that in normal man the response of cardiac muscle to an abnormal increase in filling pressure and blood volume is not of a sufficient degree to warrant the assumption that the filling pressure is necessarily the main determinant of the level of cardiac output or cardiac work. One explanation for the findings may lie in a continuous shift of cardiac function from one Starling curve to another as filling pressure rises. The implication is strong that a mechanism of this type or some other mechanism under neurohumoral control may be of more importance to the level of cardiac function than the small variations in filling pressure which occur in normal man or patients with mild to moderate forms of cardiac disease.

CONCLUSIONS

1. Intravenous infusions of distilled water, 3 per cent glucose in water, physiologic saline solution and 6 or 8 per cent dextran in either 5 per cent glucose and water or physiologic saline solution were given at a constant rate of 25 ml. per minute for periods of 30 to 100 minutes to 31 normal subjects and 15 patients with mitral stenosis.

2. During infusions of water, physiologic saline solution and 3 per cent glucose in water in normal subjects, blood volume increased 5 to 8 per cent. No other significant changes in cardiovascular hemodynamics occurred.

3. Infusions of 6 or 8 per cent dextran solutions in normal subjects were associated with increases in blood volume of 20 to 35 per cent, rises in right heart filling pressures of 2 to 12 mm. Hg and elevations of pulmonary arterial mean pressure of 3 to 15 mm. Hg. With the greatest expansion in blood volume, cardiac and stroke output were increased 31 and 20 per cent, respectively. In seven subjects, however, increases in right heart filling pressure of 4 to 12 mm. Hg were associated with changes in cardiac output of less than 15 per cent.

4. In patients with mitral stenosis increases in blood volume of 10 to 29 per cent were associated with increases in right heart filling pressures of 1 to 10 mm. Hg and increases in pulmonary arterial mean pressures of 8 to 23 mm. Hg. The mean increase in pulmonary arterial mean pressure of 13.56 mm. Hg was twice that found in normal subjects. The increase in pulmonary arterial mean pressure was most marked in patients receiving infusions of 6 per cent dextran in physiologic saline solution, being on the average three times greater than the increase found in normal subjects. Mean values for cardiac and stroke output increased 13 and 7 per cent, respectively. In six patients cardiac output changed less than 10 per cent while right heart filling pressure increased 2 to 6 mm. Hg.

5. In all patients and in all normal subjects save one, maximum increases in blood volume and right heart filling pressure were associated with increases in right ventricular stroke work. In seven instances the increase in right ventricular work was relatively small compared to the increase in right heart filling pressure, the increase in right ventricular work being less than 40 per cent while right heart filling pressures rose 2 to 12 mm. Hg.

6. No significant relation appeared to exist in normal subjects or patients between the magnitude of the increase in right heart filling pressure and the percentage change in cardiac and stroke output or the change in right ventricular stroke work.

7. In normal subjects a significant linear relation was found between the degree of blood volume expansion and the percentage change in cardiac output, stroke output and the change in right ventricular stroke work. In patients with mitral stenosis a significant linear relation was found only between the degree of blood volume expansion and the percentage change in cardiac output.

8. The relatively small alterations in cardiac function occurring with the moderate to marked elevations of right heart filling pressure suggest the importance of factors other than filling pressure in the control of cardiac function in man.

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REFERENCES

1. Symposium on the regulation of the performance of the heart. Physiol. Rev. 1955, **35**, 90.
2. Rushmer, R. F. Applicability of Starling's law of the heart to intact, unanesthetized animals. Physiol. Rev. 1955, **35**, 138.
3. Ek, J., Bucht, H., and Werkö, L. The influence of a rapid infusion of glucose on renal dynamics and sodium excretion in patients with arterial hypertension; preliminary report. Amer. Heart J. 1954, **48**, 102.
4. Sarnoff, S. J., and Berglund E. Ventricular function. I. Starling's law of the heart studied by means of simultaneous right and left ventricular function curves in the dog. Circulation 1954, **9**, 706.
5. Warren, J. V., Brannon, E. S., Weens, H. S., and Stead, E. A., Jr. Effect of increasing the blood volume and right atrial pressure on the circulation of normal subjects by intravenous infusions. Amer. J. Med. 1948, **4**, 193.
6. Cournand, A., and Ranges, H. A. Catheterization of the right auricle in man. Proc. Soc. exp. Biol. (N. Y.) 1941, **46**, 462.
7. Lagerlof, H., and Werkö, L. Studies on the circulation in man. A technique of venous catheterization with determination of cardiac output and simul-

taneous recordings of the blood pressures, the electrocardiogram, phonocardiogram, and respiration. Acta med. scand. 1949, **132**, 495.

8. Lagerlof, H., and Werkö, L. Studies on the circulation of blood in man. VI. The pulmonary capillary venous pressure pulse in man. Scand. J. clin. Lab. Invest. 1949, **7**, 147.
9. Thomasson, B. Cardiac output in normal subjects under standard basal conditions. The repeatability of measurements by the Fick method. Scand. J. clin. Lab. Invest. 1957, **9**, 365.
10. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
11. Ek, J. The influence of heavy hydration on the renal function in normal and hypertensive man. Scand. J. clin. Lab. Invest. 1955, **7**, Suppl. 19.
12. Werkö, L. The influence of positive pressure breathing on the circulation in man. Acta med. scand. 1947, Suppl. 193.
13. Harvey, R. M., Ferrer, M. I., Cathcart, R. T., Richards, D. W., Jr., and Cournand, A. Some effects of digoxin upon the heart and circulation in man. Amer. J. Med. 1949, **7**, 439.
14. Witham, A. C., Fleming, J. W., and Bloom, W. L. The effect of the intravenous administration of dextran on cardiac output and other circulatory dynamics. J. clin. Invest. 1951, **30**, 897.
15. Doyle, J. T., Wilson, J. S., Estes, E. H., and Warren, J. V. The effect of intravenous infusions of physiologic saline solution on the pulmonary arterial and pulmonary capillary pressure in man. J. clin. Invest. 1951, **30**, 345.
16. McMichael, J. Circulatory failure studied by means of venous catheterization. Advanc. intern. Med. 1947, **2**, 64.
17. Henry, J. P., Gauer, O. H., and Sieker, H. O. The effect of moderate changes in blood volume on left and right atrial pressures. Circulat. Res. 1956, **4**, 91.
18. Fowler, N. O., Franch, R. H., and Bloom, W. L. Hemodynamic effects of anemia with and without plasma volume expansion. Circulat. Res. 1956, **4**, 319.
19. Eliasch, H. Pulmonary circulation at rest and on effort in mitral stenosis. Scand. J. clin. Lab. Invest. 1952, **4**, Suppl. 4.
20. Sunahara, F. A., and Beck, L. Cardiovascular effects of acutely produced anemia in the normal dog. Amer. J. Physiol. 1954, **176**, 139.
21. Brannon, E. S., Merrill, A. J., Warren, J. V., and Stead, E. A., Jr. The cardiac output in patients with chronic anemia as measured by the technique of right atrial catheterization. J. clin. Invest. 1945, **24**, 332.
22. Berglund, E., Sarnoff, S. J., and Isaacs, J. P. Ventricular function: Role of the pericardium in regulation of cardiovascular hemodynamics. Circulat. Res. 1955, **3**, 133.
23. Albert, R. E., Smith, W. W., and Eichna, L. W. Hemodynamic changes associated with fluid retention induced in noncardiac subjects by corticotropin (ACTH) and cortisone; comparison with the hemodynamic changes of congestive heart failure. Circulation 1955, **12**, 1047.
24. Holmgren, A. Circulatory changes during muscular work in man. with special reference to arterial and central venous pressures in the systemic circulation. Scand. J. clin. Lab. Invest. 1956, **8**, Suppl. 24.
25. Asmussen, E., and Nielsen, M. Cardiac output during muscular work and its regulation. Physiol. Rev. 1955, **35**, 778.
26. Cournand, A., Motley, H. L., Werkö, L., and Richards, D. W., Jr. Physiological studies of the effects of intermittent positive pressure breathing on cardiac output in man. Amer. J. Physiol. 1948, **152**, 162.
27. McMichael, J., and Sharpey-Schafer, E. P. Cardiac output in man by direct Fick method. Effects of posture, venous pressure change, atropine, and adrenaline. Brit. Heart J. 1944, **6**, 33.
28. Stead, E. A., Jr., Warren, J. V., Merrill, A. J., and Brannon, E. S. Cardiac output in male subjects as measured by the technique of right atrial catheterization. Normal values with observations on the effect of anxiety and tilting. J. clin. Invest. 1945, **24**, 326.
29. Hickam, J. B., and Pryor, W. W. Cardiac output in postural hypotension. J. clin. Invest. 1951, **30**, 401.
30. Starling, E. H. The Linacre Lecture on the Law of the Heart. (Cambridge, 1915.) London, Longmans, Green and Co., 1918.

SOME FACTORS AFFECTING INDICATOR DILUTION CURVES IN THE PRESENCE AND ABSENCE OF VALVULAR INCOMPETENCE

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The indicator dilution technique of Stewart was reintroduced in practicable form by Kinsman, Moore and Hamilton in 1929 (1), was developed further by this group (2, 3), and since then has been extensively used to measure cardiac output and central blood volume (the volume of blood between the site of injection and sampling of the indicator). In 1951 Kopelman and Lee (4) observed that tricuspid regurgitation greatly prolonged the time concentration curve, and more recently Korner and Shillingford (5, 6) by analysis of data from model experiments attempted to show that the degree of alteration of the curve was proportional to the amount of regurgitant flow.

This principle, that regurgitant flow can be quantitatively or semiquantitatively estimated from indicator dilution curves, has been used recently both for clinical and for physiological studies. Clinically, it has been evaluated as an additional test for estimating the severity of mitral incompetence (7–10); physiologically, it has been used to determine the effects of rest and exercise on tricuspid incompetence (11) and to clarify the role of valvular incompetence in heart failure (12). Underlying all these studies is the assumption that changes in the indicator dilution curve depend only on the cardiac output, central volume and amount of regurgitation. But incompetence of a cardiac valve may be associated with many other hemodynamic changes: The stroke volume and/or heart rate may increase; the distribution of blood volume behind and in front of the incompetent valve may alter; the heart chambers and great veins may change in capacity; and distention or disease may alter the elasticity of the atria, ventricles or great vessels and thereby affect streaming and mixing of indicator, particularly in the regurgitant jet. Not all of these changes could be controlled or assessed in the model which Korner and Shillingford used (5, 6), and a new model was therefore devised to assess the influence of some of these changes on indicator dilution curves.

MATERIAL AND METHODS

A rigid model was designed to allow direct measurement of forward and regurgitant flow (Figure 1). In this model the volume upstream (proximal) and downstream (distal) to the valves could be altered by inserting a glass sphere at S_1 or S_2; the stroke volume could be changed from 20 to 42 ml. by moving the attachment of the piston to the cam; the residual volume of the pump could be changed from 0 to 45 ml. by altering the distance between the barrel of the syringe and the cam; and the valve V_3 could be made incompetent. When it was incompetent dye dilution curves were made with three types of chamber at S_1: *1*) a 235 ml. sphere with its entrance and exit at 90° to each other (C_1); *2*) a 235 ml. sphere with its entrance and exit at 180° to each other (C_2); and *3*) the 235 ml. sphere placed at S_2 so that the chamber before the pump consisted of the 2 cm. wide tubing of the model (C_3). During experiments on valvular incompetence the aperture of reflux was constant, and forward and regurgitant flows were regulated by changing the pump rate and the resistances to each outflow. Both forward and backward outflow (at B_2 and B_1) were collected simultaneously for 30 seconds during the inscription of the dye dilution curve and were measured in the same cylinder to the nearest 5 ml.

In some experiments a thin rubber segment dilated to form a distensible chamber (E) of variable size was inserted at S_1, and valves V_1 and V_2 were removed. With each regurgitant jet the elastic chamber dilated and allowed the jet to mix with the incoming water although some of the water in the system was displaced backwards into the reservoir which was open to air. In this elastic system it was no longer possible to measure the regurgitant flow directly, but it could be estimated by subtracting the measured forward output from the total minute out-

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put of the pump. This estimate is approximate because during the downstroke of the piston a small amount of water, found in preliminary experiments with competent valves not to exceed 2 to 4 ml. per stroke, flows through the model. As a result the measured forward output is slightly greater than the calculated output of the pump, and the backflow would be underestimated by this method of calculation.

Evans blue dye (T-1824) was injected rapidly at point I with an insulin syringe fitted with a metal barrel cap to limit withdrawal of the plunger. This syringe could therefore be filled with the same amount each time, and the amount of dye injected (1 mg. per ml.) was found by weighing to be 1.6873 ± 0.019 mg. After each injection the pump was run until all the dye had been washed out of the model and the curve had returned to the base line.

The dye curves were made with a photo cell ear piece oximeter and amplifier (5) and an Evershed direct-writing recorder. As the response was alinear a calibration curve was made from a series of dye solutions of concentration 0.5 to 4.0 mg. per L. and the heights of the dye curve at one second intervals were converted by this calibration curve into concentrations in mg. per L. The sensitivity of the system was rechecked after every five to six curves and appropriate corrections were made when necessary.

Five curve parameters and two ratios were studied: *1*) slope (S); *2*) variance (s^2); *3*) the time taken for the dye concentration to fall to 0.1 mg. per L. (PT); *4*) appearance time (AT); *5*) mean time of the curve (MTT) calculated from the sum of dye concentrations at each second throughout the curve times each corresponding time interval divided by the sum of dye concentration at each second throughout the curve (SCT/SC); and the ratios PT/AT and PT/MTT.

All outputs referred to are those measured directly, and these were also used in the calculation of central volume. This was calculated according to the methods of Hamilton, Moore, Kinsman and Spurling (3) but modified by fitting the downslope to the first 8 to 10 points of the straight line descent by the method of least squares (13) and by summing the dye concentrations to infinity (14). These modifications were also used in calculating the variance of the curve. Experimental differences between parameters or ratios were evaluated by an analysis of regression technique, the adjusted mean being compared by weighting the variances of the groups (15).

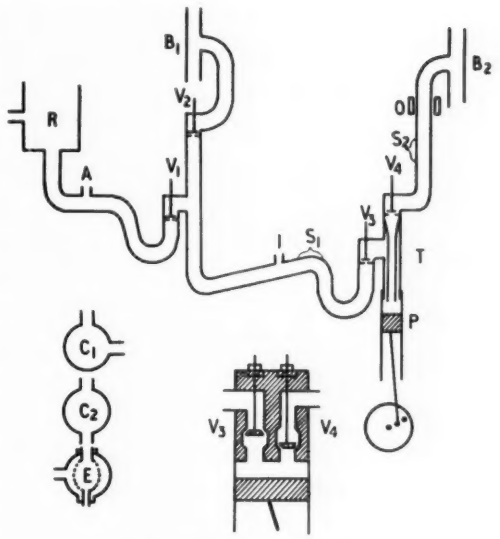

FIG. 1. DIAGRAM OF THE MODEL

The diagram is labeled as follows: A, inflow from tap; B_1 and B_2, outflow tubes for measuring forward and backward flow, open to air to prevent siphoning; O, oximeter earpiece; I, site of dye injection; P, piston pump (100 ml. syringe). Piston attached to a cam run by a variable speed motor; R, reservoir (with spillover to keep head of pressure constant) kept below level of outflow tubes to prevent continuous forward flow; S_1 and S_2, sites at which the volume of the apparatus could be altered or an elastic chamber inserted; C_1 and C_2, glass spheres inserted at S_1 and S_2; E, elastic segment inside glass sphere inserted at S_1; V_{1-4} gravity valves; and T, tube to eliminate dead space.

In some experiments other valves (inset lower center) were substituted for V_3 and V_4. When the valves were competent water was pumped out at B_2 with little or no spillover (not more than 20 ml. per minute) through V_2 and out at B_1; this small loss was caused by the pressure wave set up each time V_3 closed and seemed proportional to the force of its closure. When V_2 was incompetent, with the rigid model, each regurgitant jet displaced an equal amount of water past V_2 and out of B_1; the site of the injection of dye was far enough forward to prevent loss of dye at B_1, and B_1 was higher than B_2 to prevent outflow in the absence of incompetence of V_3.

RESULTS [1]

A. Rigid model, no valvular incompetence

Dye dilution curves were made with stroke volumes of 20 and 42 ml. and residual volumes of 0 and 45 ml. To determine how each curve parameter and ratio was related to output and central volume the data from each combination of stroke and residual volume were pooled and used to calculate the correlation coefficients and partial correlation coefficients *1*) between each curve parameter (or ratio) and output and *2*) between each curve parameter (or ratio) and central volume. Logarithms of output, central

[1] Copies of supplementary tables are available at the Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C.

TABLE I

Relationships between curve parameters or ratios, output and central volume in rigid systems without regurgitation *

Parameter or ratio	Output			Log. central volume					
	r_{13}	$r_{12.3}$	Significance of $r_{12.3}$	r_{13}	$r_{12.3}$	Significance of $r_{12.3}$	r	r^2	$1-r^2$
Slope (S)	+0.91	+0.92	$0.001>p$	+0.49	−0.59	$0.001>p$	0.94	0.89	0.11
Appearance time (AT)	+0.18	−0.77	$0.001>p$	+0.74	+0.90	$0.001>p$	0.91	0.82	0.18
Mean time of curve (MTT)	−0.85	−0.91	$0.001>p$	−0.37	+0.69	$0.001>p$	0.93	0.86	0.14
Ratio duration/mean time of curve (PT/MTT)	−0.47	−0.20	$0.4>p>0.3$	−0.48	−0.24	$0.3>p>0.2$	0.51	0.26	0.74
	Log. output			Log. central volume					
	r_{13}	$r_{12.3}$	Significance of $r_{12.3}$	r_{13}	$r_{12.3}$	Significance of $r_{12.3}$	r	r^2	$1-r^2$
Log. variance (log s^2)	−0.92	−0.93	$0.001>p$	−0.49	+0.57	$0.005>p>0.001$	0.94	0.89	0.11
Log. duration (log PT)	−0.92	−0.94	$0.001>p$	−0.47	+0.63	$0.001>p$	0.95	0.90	0.10
Log. ratio (PT/AT)	−0.69	−0.25	$0.2>p>0.1$	−0.85	−0.71	$0.001>p$	0.86	0.74	0.26

* The correlation coefficient between each parameter and the output is r_{12}, and between each parameter and the central volume, r_{13}. The partial correlation coefficient between each parameter and the output when the central volume is constant is $r_{12.3}$, and between each parameter and the central volume when the output is constant, $r_{13.2}$. The multiple correlation coefficient is r; r^2 is the proportion of the observed variation in the parameter under consideration which can be attributed to the effects of output and central volume and $1-r^2$ that portion which may be attributed to other factors.

volume or of the parameter or ratios were used if preliminary calculations showed that a higher correlation could be obtained thereby. The multiple correlation coefficient (r) between all three is also given (Table I).

All the curve parameters (but not the ratios) were largely dependent on output and central volume. The small residual variation ($1-r^2$) of less than 20 per cent was due to factors other than changes in output and central volume, and included differences resulting from changes in stroke and residual volume as well as from experimental error. Comparisons of the four combinations of stroke and residual volume showed no difference between the slopes of any regression planes except those for the ratio PT/MTT ($0.005 > p > 0.001$). The means of each parameter or ratio adjusted to the same output and central volume are given in Table II, as are the relevant variance ratios and estimates of significance. The adjusted mean values in the table were obtained by *a*) averaging the forward outputs and central volumes from both groups being compared and then *b*) using separate regression formulae for each chamber to adjust the mean value of the parameter or ratio to these averages. In effect this shows what the mean values would have been had all the experiments been made at a constant output and central volume. The values for C_1 differ in the two comparisons because the outputs and central volumes used in the experiments with chambers C_2 and C_3 were not identical so that the averages they make with the values obtained from the experiments with chamber C_1 differ slightly. The adjusted group means showed statistically significant differences for all except slope and variance, although the actual differences were small (*e.g.*, adjusted mean curve times varied from 14.2 to 14.8 seconds) and were not apparent from careful inspection of the individual curves.

B. Rigid model, valvular incompetence

In all these experiments (the basic data of which is available in supplementary tables) the amount of regurgitant flow and its ratio to the total flow were approximately constant. In each group the forward output varied from 1.5 to 1.9 L. per minute; the backflow varied from 65 to

TABLE II

Effect of stroke and residual volume in rigid systems without regurgitation. Comparisons of adjusted means *

Stroke volume (ml.) / Residual volume (ml.)	42 / 0	42 / 45	20 / 0	20 / 45	Variance ratio F	Estimate of significance†
Slope (S)	0.1011	0.1006	0.0974	0.1008	1.68	$0.3>p>0.1$
Appearance time (AT)	10.7	9.5	10.1	10.0	24.10	$0.0005>p$
Mean time of curve (MTT)	14.2	14.8	14.6	14.3	5.00	$0.025>p>0.01$
Duration (PT)	41.5	43.5	42.7	42.6	5.27	$0.025>p>0.01$
Variance (s^2)	102.9	108.4	111.4	106.9	1.90	$0.3>p>0.1$
PT/AT	3.90	4.59	4.21	4.30	13.58	$0.0005>p$
PT/MTT	3.05	2.94	3.00	2.98	23.37	$0.0005>p$

* The means of each of the parameters and ratios were adjusted mathematically to the same output and central volume to allow statistical comparisons between them.

† The estimate of significance gives the probability that all four means came from the same population, *i.e.*, were not affected by changes in stroke and residual volumes. There are small but significant changes in each parameter (and ratio) except slope and variance, and even these showed trends toward change.

80 per cent of the forward output and the central volume ranged from 660 to 736 ml. It was therefore possible to determine if the effect of valvular incompetence in changing the curve parameters was dependent on alterations of the chamber proximal to the incompetent valve. Regression equations relating each parameter or ratio to output and central volume were calculated for each type of proximal chamber (sphere with entrance and exit at 90° to each other—C_1; sphere with entrance and exit at 180° to each other—C_2; tube—C_3) and statistical comparisons were made between these regression equations from C_1 and C_2 and from C_1 and C_3. No differences were shown between the slopes of the regression planes of any of the parameters or ratios except PT/MTT ($0.05 > p > 0.03$) and this only in the comparison of C_1 and C_3. There were, however, significant differences between the adjusted means of some of the parameters and ratios (Table III).

Curves A, B and C in Figure 2 show dia-

TABLE III

A. Comparison of adjusted means of parameters and ratios from curves made with the rigid model with regurgitant flow obtained for proximal chambers C_1 and C_2 *

Parameter	C_2	C_1	Variance ratio F	Estimate of significance
Slope	0.0820	0.0856	7.07	$0.05>p>0.025$
Appearance time (AT)	8.4	8.5	0.02	$p>0.5$
Mean time of curve (MTT)	16.5	16.3	0.27	$p>0.5$
Duration (PT)	49.0	48.0	3.05	$0.3>p>0.1$
Variance	155.2	143.3	7.37	$0.05>p>0.025$
PT/AT	5.86	5.74	0.47	$p>0.5$
PT/MTT	2.98	2.93	2.14	$0.3>p>0.1$

B. Comparison of adjusted means obtained for proximal chambers C_1 and C_3 *

Parameter	C_1	C_3	Variance ratio F	Estimate of significance
Slope	0.0848	0.0922	7.91	$0.05>p>0.025$
Appearance time (AT)	8.6	9.5	7.64	$0.05>p>0.025$
Mean time of curve (MTT)	16.5	15.7	6.91	$0.05>p>0.025$
Duration (PT)	48.6	45.4	8.92	$0.025>p>0.01$
Variance	147.6	123.4	13.94	$0.01>p>0.005$
PT/AT	5.67	4.83	7.82	$0.05>p>0.025$
PT/MTT	2.94	2.93	0.06	$p>0.5$

* The means of each parameter and ratio have been adjusted to the same central volume and output. The central volume and output used in each part of the table is the average for the observations in which the two proximal chambers under consideration were used. This average differs somewhat for C_1 *vs.* C_2 as compared with C_1 *vs.* C_3 and explains why C_1 values are not the same in each part of the table.

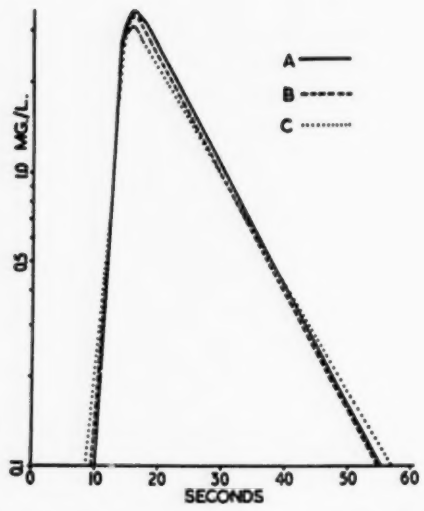

FIG. 2. COMPOSITE DIAGRAM TO DEMONSTRATE THE DIFFERENCE PRODUCED BY VALVULAR INSUFFICIENCY IN A RIGID MODEL

For explanation see text.

grammatically the differences between dye dilution curves made when there was no valvular incompetence and those made with proximal chambers C_1 and C_3 when there was backflow. These composite curves were constructed from separate prediction formulae for each group by adjusting statistically to a constant output of 1.68 L. per minute and central volume of 700 ml. the means of the slopes (S), appearance times (AT) and the times taken for the dye concentrations to fall to 0.1 mg. per L. (PT). In each composite curve the maximum dye concentration and time at which it occurred is approximate so that the portion of each curve from its appearance to the beginning of the straight line downslope is hypothetical. Curve A was derived from 21 curves made when there was no valvular incompetence, curve B was derived from six curves made with the small proximal chamber C_3 when the backflow averaged 75 per cent of the forward flow, and curve C was derived from six curves made with the large proximal chamber C_1 when the backflow averaged 73 per cent of the forward flow. In all the stroke volume was 42 ml.; the residual volume was 0 ml. in half the experiments done in each group and 45 ml. in the other half.

Valvular incompetence has made the appearance time shorter, the duration of the curve longer, the maximum dye concentration less and the slope smaller. All of these changes were less marked for small proximal chamber C_3 (curve B) than for the large proximal chamber C_1 (curve C), and despite the relatively large backflows neither composite curve differs greatly from the normal.

The mean circulation time is the sum of the appearance time and the mean time of the curve, both of which may change when there is valvular incompetence. To see if this affected the calculated central volume, analyses were made of dye dilution curves obtained with and without valvular incompetence but with no change in the physical volume of the model. The calculated central volume in these experiments was unchanged by valvular incompetence, but the distortion of the curves was small (Figure 2); it was therefore possible for the shortened appearance time to balance the longer mean time of the curve. With more marked curve distortion this may not occur since the mean time of the curve can be greatly prolonged and it is difficult to see how the appearance time could shorten enough to compensate for this.

C. Elastic model, valvular incompetence

Table IV gives the statistics for dye dilution curves made with and without valvular incompetence and with small and large elastic proximal chambers. In all experiments the stroke volume was 42 ml. and there was no residual volume in the pump. When the valves were competent the dye dilution curves were similar to, though perhaps slightly less prolonged than, those obtained from the rigid model at similar outputs and central volumes; two few experiments were done with the elastic proximal chamber to test this difference rigorously.

The magnitude of the change in the parameters of the dye dilution curves when valvular incompetence was introduced was related to the size of the proximal elastic chamber. In Experiments A and B, illustrated in Figure 3 and marked in Table IV, the forward output and central volume were the same, the regurgitant flow was 2.99 L. per minute in A and 2.40 L.

TABLE IV

Effect of valvular incompetence and an elastic chamber on dye curves

Forward flow	Back-flow	Rate/min.	Central volume	Size of proximal chamber*	Slope	Variance	Duration (PT)	Appear-ance time (AT)	Mean time of curve (MTT)	PT/AT	PT/MTT	
L./min.	*L./min.*		*ml.*				*sec.*	*sec.*	*sec.*			
2.18		52	748	S	0.1425	52.3	31.5	10.0	10.6	3.15	2.97	
1.56		44	759	S	0.0974	110.1	46.1	13.0	16.3	3.55	2.83	C
1.80		44	768	S	0.1097	87.1	40.0	12.2	13.4	3.28	2.99	
1.86		44	786	S	0.1167	77.2	38.7	12.0	13.4	3.23	2.89	
1.58		36	1,282	L	0.0403	641.5	88.0	14.8	33.9	5.95	2.60	
1.55	2.99	108	850	S	0.0903	137.2	50.4	14.4	18.5	3.50	2.72	A
1.55	2.40	94	847	L	0.0552	334.0	67.3	9.9	22.9	6.80	2.94	B
1.58	1.78	80	919	S	0.0737	196.9	58.7	13.5	21.4	4.35	2.74	

* S designates small proximal chamber; L, large proximal chamber (capacity about 400 to 500 ml.). Changes in volume of the proximal chamber, with no change in forward output and central volume, have allowed regurgitation to produce gross alterations in curve parameters and ratios. The introduction of an elastic system has made changes, previously detected only by mathematical analysis, obvious.

per minute in B, and the proximal elastic chamber was 235 ml. smaller in A than in B. Despite the smaller regurgitant flow in B, the alterations of its curve parameters and ratios were much greater than those in A.

DISCUSSION

It has been recognized since 1932 (3) that the downslope of indicator dilution curves (made without regurgitant flow) was determined largely by output and central volume. This was confirmed by statistical analyses of data obtained from a circulation model and man by Korner and Shillingford (5, 6), who also demonstrated that other curve parameters (variance, mean time of the curve) were similarly determined and could be predicted for any output and central volume.

In the present study changes in stroke and residual volume were also shown to have significant effects on the parameters of dye dilution curves. These effects were, however, small (accounting for less than 20 per cent of the total variation, Tables I and II), in spite of the fact that the groups compared ranged from those in which the pump emptied completely or almost completely at each stroke to those in which the residual volume was more than double the stroke volume. These findings do not confirm the theory of Comét and Lagerlöf (16) that the downslope of the indicator-dilution curve is determined by the left ventricular stroke and residual volume and that this relationship can be used to calculate the residual volume of the left ventricle. An exponential washout slope dependent on the ratio $\frac{\text{residual volume}}{\text{stroke} + \text{residual volume}}$ would indeed be expected if the indicator were injected into the left ventricle, mixed completely with the left ventricular blood and sampled just above the aortic valve; experimental evidence for this has been presented by Holt (17). If, however, the indicator is injected or sampled far from the ventricle (and it was for this type of curve that Comét and Lagerlöf made their mathematical analysis) the relationships between stroke volume, residual volume and the slope are obscured by the washout slope imposed by the additional

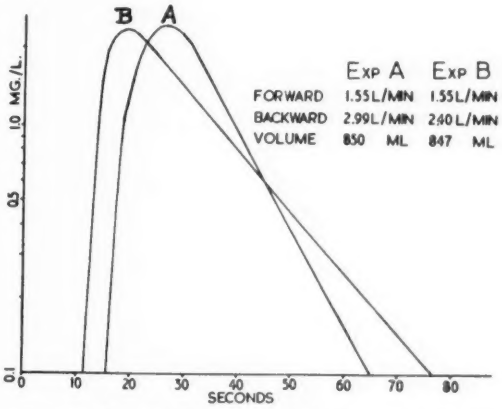

FIG. 3. DYE CURVES MADE WITH SMALL AND LARGE ELASTIC PROXIMAL CHAMBERS AND VALVULAR INCOMPETENCE

TABLE V

Comparison of backflow observed and estimated by three methods of calculation in the rigid model

	Residual volume	Forward flow	Backflow (*L./min.*)*			
				Calculated		
			Observed	Predicted from slope	Ratio of variances	Ratio of slopes
	ml.	*L./min.*				
Tube C_2	0	1.57	1.24	−0.06	−0.12	−0.07
	0	1.58	1.22	0.01	0.07	0.01
	0	1.80	1.45	−0.05	−0.07	−0.06
	45	1.68	1.30	0.15	0.34	0.24
	45	1.73	1.26	0.01	−0.08	0.02
	45	1.78	1.19	0.13	0.20	0.17
Sphere C_1	0	1.60	1.28	0.08	0.23	0.10
	0	1.66	1.14	0.12	0.36	0.17
	0	1.68	1.20	0.13	0.32	0.16
	45	1.63	1.13	0.17	0.35	0.24
	45	1.66	1.21	0.20	0.44	0.30
	45	1.78	1.11	0.20	0.41	0.27

* Comparison of observed and calculated backflow shows how far off from the actual backflow the calculated amount may be; the amount of error bears little relation to the method used.

central volume. The corollary of this, namely that despite a constant left ventricular stroke and residual volume the downslope changes as the central volume changes, was shown by Hetzel, Swan and Wood (18). They injected dye successively at different sites (pulmonary artery, right atrium, superior vena cava and brachial vein) and found that the curve became lower and wider as the site of injection moved peripherally. Since cardiac output and rate varied little between successive injections and since residual ventricular volume was not likely to have altered much, the changes of slope were therefore related to differences of central volume and independent of stroke and residual volume. Thus, in practice left ventricular stroke and residual volume probably have little effect on the parameters of indicator dilution curves, although if the central volume is small relative to the stroke and residual volume, *e.g.*, with injection into the left atrium or left ventricle, it may not be safe to assume that curve parameters are related only to output and central volume.

With valvular regurgitation varying changes have been found in curve parameters when, with similar amounts of backflow, the type of proximal chamber has been altered; these differences were present even when allowance was made for varia-

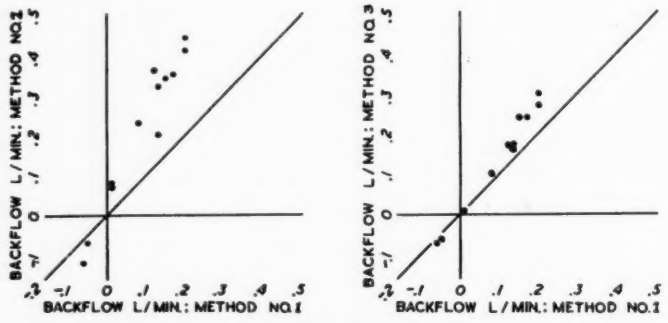

FIG. 4. COMPARISONS OF BACKFLOWS ESTIMATED BY DIFFERENT METHODS

Method 1: From observed slope reciprocal (5). Method 2: From ratio of predicted to observed variances (6). Method 3: From ratio of predicted to observed slope reciprocals (8, 19).

tion in output and central volume (Tables III and IV). As a result the estimates of backflow by existing methods of calculation (5, 6, 8, 10) varied with the type of proximal chamber, and comparisons between the backflows observed and calculated by three methods are given in Table V. Not only do these estimates differ in rigid systems for the different types of proximal chamber and perhaps for different residual volumes, but they all greatly underestimate the actual backflow. In addition the three methods do not give similar estimates of the same backflow (Figure 4).

In the absence of a sufficiently large series of experiments done without valvular incompetence and with an elastic proximal chamber it was not possible to make a regression equation for predicting curve parameters from output and central volume. It is possible, however, to set limits for these parameters. In Experiment A (Table IV) the upper and lower limits of the slope predicted without backflow are 0.0974 (found in Experiment C for the same output but a smaller central volume) and 0.0903 (found in Experiment A itself with 2.99 L. per minute backflow); corresponding limits of the predicted curve variance can be determined so that the upper and lower limits of the ratios of predicted and observed slopes and variances can be calculated, and backflows can be estimated (5, 6, 8, 10). The backflow of 2.99 L. per minute in Experiment A is estimated from the ratio of slopes as 0 to 0.12 L. per minute and from the ratio of variances as 0 to 0.38 L. per minute; in Experiment B the backflow of 2.40 L. per minute is estimated as 0.98 to 1.18 L. per minute from the ratio of the slopes and 2.23 to 3.16 L. per minute from the ratio of the variances. It is important to note that estimates by these two methods differ greatly, that the actual backflow in Experiment A was grossly underestimated by both methods, and that an increase of 235 ml. in the proximal elastic chamber has increased the estimated backflow by about 1 L. per minute (slope method) or by about 2 to 3 L. per minute (variance method), even though the actual backflow had decreased. An equivalent increase in the volume of the proximal chamber in the rigid model changed the estimated backflow by about 20 to 40 ml. (Table V), a relatively small amount even allowing for the smaller backflow in these experiments.

These observations may best be explained by the hypothesis that most of the changes in the parameters of the curves are due not only to the amount of backflow but also to the dilution of the regurgitated dye. The extent to which dilution occurs may depend upon the volume and shape of the proximal chamber; its elasticity; the force, shape and direction of the regurgitant jet; and swirling and streaming of fluid within the proximal chamber. If the regurgitated dye mixes poorly with the fluid in the proximal chamber, *e.g.*, in the rigid model with the small proximal chamber, it will be little diluted and will rapidly be washed into and out of the pump, so that little distortion of the curve will result. If, on the other hand, the regurgitated dye mixes well with a large volume of fluid in the proximal chamber, *e.g.*, in the model with the large elastic chamber, it will be greatly diluted, its washout will consequently be prolonged and the curve will be greatly distorted. The more the dilution of a given amount of regurgitant dye the lower will be the maximum and mean concentrations of dye at the sampling site. Differences between chambers C_1 and C_2 may have been due to streaming, for it has been shown that the positions of the entrance to and exit from a sphere influence the passage of dye through it (19).

This hypothesis has important implications in man, for valvular disease may be associated with great alterations in size, shape and elasticity of the atria and ventricles, and in the force, shape and direction of the regurgitant jet. For example, since the residual volume of blood in the left ventricle in aortic incompetence is probably less than that in the right atrium for an equal amount of tricuspid incompetence, the dilution of regurgitated dye may be less with the aortic lesion and so produce less alteration of the curve parameters. In disease of the mitral valve, too, great variation of left atrial size is well known, calcification of the wall may decrease elasticity and thrombi may influence mixing. Examples of inadequate mixing of dye and blood in the left atrium have been cited by Woodward, Swan and Wood (20). Though it has not been determined how these changes influence curve parameters it is possible

that they may cause the parameters to vary independently of the amount of backflow and so make difficult its assessment. Finally, in experimental procedures (*e.g.*, rest, exercise, digitalization) it may be difficult to assess not only the amount of backflow but even whether it increases or decreases, for once again the differences produced in the curve parameters may be due to changes in the dilution of the regurgitated dye as well as to its amount.

Parameters and ratios from indicator dilution curves have also been compared with assessments of mitral incompetence made by routine clinical and laboratory methods, and have been found to alter as the severity of mitral incompetence increased (*7*, 9). These methods do not attempt to estimate absolute amounts of backflow, but are necessarily limited in their value by the accuracy of the criteria used to define the severity of the mitral insufficiency and the extent to which the parameters and ratios used are altered by variables other than the amount of regurgitation.

This study has thus shown that several factors other than output, central volume and the amount of backflow may influence the parameters of indicator dilution curves. If the only major variables in a model are forward output, central volume and backflow, it will be possible to construct formulae for relating these to the curve parameters and thus, knowing forward output and central volume, to estimate the amount of backflow. This estimate would not necessarily be correct in different models with additional variables, although if these could be measured it should be possible to construct formulae which take them also into account. It is difficult to be sure that any model successfully reproduces all the variables found in the human, and therefore the extension of formulae derived from any model to calculate the backflow in man should be validated by independent estimates of that backflow in man himself.

SUMMARY

Dye dilution curves were made in a circulation model in which the amount of forward output and of backward flow through an incompetent valve could be measured directly in a calibrated flask. When the valves were competent, all the curve parameters examined (slope, variance, duration, appearance time and mean time of the curve) were markedly dependent on output and central volume. When the stroke volume of the pump was changed from 20 to 42 ml. and its residual volume from 0 to 45 ml. there were slight changes of these parameters as well as of the ratios of the curve duration to *1*) the appearance time and *2*) the mean time of the curve; these changes were statistically significant for all but slope and variance, and both of these showed trends towards change. It is probable that the changes were small because the central volume was large relative to the stroke and residual volume.

When a valve was incompetent changes were made in the size, shape and elasticity of the proximal chamber entered by the regurgitant jet, and the alterations produced in the curve parameters and ratios by similar amounts of backflow depended in part on these changes. With a narrow and rigid proximal chamber there was little distortion of the curve despite a relatively large backflow, whereas with a large elastic proximal chamber gross distortion of the curve occurred. Although the type of change in the curve was that described by Korner and Shillingford (5, 6) (*i.e.*, earlier appearance time, lower and longer curve), backflow estimated in our model by their formulae could differ greatly from the measured backflow and varied with the type of proximal chamber.

The hypothesis is made that the distortion of the indicator dilution curve produced by backflow is due to the dilution and subsequent washout of the regurgitated indicator and that this depends not only on the amount of backflow but also on the nature of the regurgitant jet and the chamber which it enters.

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REFERENCES

1. Kinsman, J. M., Moore, J. W., and Hamilton, W. F. Studies on the circulation. I. Injection method: Physical and mathematical considerations. Amer. J. Physiol. 1929, **89**, 322.
2. Moore, J. W., Kinsman, J. M., Hamilton, W. F., and Spurling, R. G. Studies on the circulation. II. Cardiac output determinations; comparison of the injection method with the direct Fick procedure. Amer. J. Physiol. 1929, **89**, 331.
3. Hamilton, W. F., Moore, J. W., Kinsman, J. M., and Spurling, R. G. Studies on the circulation. IV. Further analysis of the injection method, and of changes in hemodynamics under physiological and pathological conditions. Amer. J. Physiol. 1932, **99**, 534.
4. Kopelman, H., and Lee, G de J. The intrathoracic blood volume in mitral stenosis and left ventricular failure. Clin. Sci. 1951, **10**, 383.
5. Korner, P. I., and Shillingford, J. P. The quantitative estimation of valvular incompetence by dye dilution curves. Clin. Sci. 1955, **14**, 553.
6. Korner, P. I., and Shillingford, J. P. Further observations on the estimation of valvular incompetence from indicator dilution curves. Clin. Sci. 1956, **15**, 417.
7. Woodward, E., Jr., Burchell, H. B., and Wood, E. H. Dilution curves associated with valvular regurgitation. Proc. Mayo Clin. 1957, **32**, 518.
8. Novack, P., Schlant, R. C., Haynes, F. W., and Phinney, A. O., Jr. The hemodynamics of mitral valvular disease (abstract). J. clin. Invest. 1957, **36**, 917.
9. Shillingford, J. P., and Zoob, M. Dye dilution curves in the clinical assessment of mitral valve disease. Brit. Heart J. 1957, **19**, 589.
10. Novack, P., Schlant, R. C., Phinney, A. O., Jr., Haynes, F. W., and Dexter, L. Korner-Shillingford method for measurement of valvular insufficiency. Fed. Proc. 1957, 16(1), 95.
11. Korner, P., and Shillingford, J. Tricuspid incompetence and right ventricular output in congestive heart failure. Brit. Heart J. 1957, **19**, 1.
12. McMichael, J., and Shillingford, J. P. The role of valvular incompetence in heart failure. Brit. med. J. 1957, **1**, 537.
13. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 4th ed. Ames, Iowa, Collegiate Press, 1946.
14. Hoffman, J. I. E. The calculation of output, central volume and variance from indicator dilution curves. To be published.
15. Kendall, M. G. The Advanced Theory of Statistics, 3rd rev. ed. London, England, Charles Griffin and Co., 1948, p. 244.
16. Comét, S., and Lagerlöf, H. A mathematical analysis of indicator dilution curves leading to direct calculation of the systolic and diastolic volumes of the left ventricle and auricle and of the pulmonary blood volume. Acta physiol. scand. 1956, **36**, 337.
17. Holt, J. P. Estimation of the residual volume of the ventricle of the dog's heart by two indicator dilution techniques. Circulat. Res. 1956, **4**, 187.
18. Hetzel, P. S., Swan, H. J. C., and Wood, E. H. Influence of injection site on arterial dilution curves of T-1824. J. appl. Physiol. 1954, **7**, 66.
19. Hoffman, J. I. E., and Shillingford, J. P. Minor factors influencing indicator dilution curves. Clin. Sci. 1957, **16**, 1.
20. Woodward, E., Jr., Swan, H. J. C., and Wood, E. H. Evaluation of a method for detection of mitral regurgitation from indicator-dilution curves recorded from the left atrium. Proc. Mayo Clin. 1957, **32**, 525.

PLATELETS AS A SOURCE OF SERUM ACID NITROPHENYLPHOSPHATASE *

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Recent interest in serum enzyme activity in pathological conditions has led to renewed curiosity concerning the source of the enzymes present in serum under normal conditions. Since blood platelets contain acid phosphatase (1–8), it seemed possible that they might liberate this enzyme during blood coagulation. This possibility was investigated by comparing the acid phosphatase activity of serum prepared from platelet-rich and platelet-poor plasmas. Preliminary studies suggested that the platelets contribute virtually all of the activity of normal serum against β-glycerophosphate at acid pH (9). The present investigation was carried out using *p*-nitrophenylphosphate as substrate.

METHODS

Acid phosphatase activity was measured using *p*-nitrophenylphosphate [1] as substrate (10–12). This method gave highly reproducible values; for example, nine out of 10 determinations on a sample of fresh serum were 0.39 μM per ml. per hour and the tenth was 0.41.

The subjects were either healthy young adults or, in a few instances, patients with metastatic carcinoma of the prostate.[2] To avoid the use of anticoagulants, native platelet-rich and platelet-poor plasmas were prepared by centrifugation of blood drawn in chilled siliconed syringes and cooled in an ice bath in siliconed tubes. Most of the red and white cells were sedimented by centrifuging for five to 10 minutes at about 1,000 rpm. Two ml. of the supernatant platelet-rich plasma was set aside in an ice bath and a platelet count taken. The remainder of the native blood and platelet-rich plasma was centrifuged in a multispeed attachment at about 10,000 rpm for 10 minutes to obtain platelet-poor plasma. About 1 ml. of each plasma was then placed in each of two glass tubes. One pair of plasmas was incubated at 37° C. and a second pair was placed at room temperature for one hour. The platelet-rich samples always clotted, but the platelet-poor samples often contained only traces of fibrin. Since spontaneous retraction did not usually occur, the clots were removed with an applicator stick at the end of incubation, and acid phosphatase activity was measured. These samples are designated as "serum from platelet-rich plasma" and "serum from platelet-poor plasma."

The time course of acid phosphatase liberation was studied by placing 0.4 ml. of native platelet-rich plasma in each of a series of new glass tubes (rinsed with distilled water and oven-dried) at 37° C. and, at intervals, removing the platelets. In the samples which had not yet clotted, this was accomplished by brief high-speed centrifugation and the fibrin which formed subsequently in the platelet-poor plasma was removed with an applicator stick. In the samples which had clotted, the clot and entrapped platelets were removed together.

To determine whether anticoagulants inhibited enzyme activity, one-fifth volume of 0.1 M sodium oxalate, 0.11 M sodium citrate, or 0.027 M (1 per cent) disodium ethylenediaminetetraacetate in saline (EDTA) was added to serum separated from native platelet-rich plasma or from whole blood. To study the effect of the anticoagulants on the color of nitrophenol, saline was used instead of serum. The activity of whole platelets was tested by comparing the acid phosphatase activity of platelet-rich and platelet-poor plasmas separated from blood in which clotting was prevented by the addition of one-ninth volume of anticoagulant. Other studies were made on washed platelets, obtained by differential centrifugation of blood collected in EDTA and washed three times with isotonic saline at 4° C. in a multispeed attachment. A platelet count of about 500,000 per cu. mm. on the saline platelet suspension was appropriate for the measurement of nitrophenylphosphatase activity when 0.2 ml. of suspension was used. All platelet counts were done with a phase microscope (13).

RESULTS

In contrast to the results with β-glycerophosphate (9), substantial activity was found in the serum from native platelet-poor plasma (Table I). Identical values were obtained whether the platelet-poor plasma was allowed to incubate at 37° C. or at room temperature, indicating that the enzyme in this serum was not very heat labile. As found with glycerophosphate, serum prepared from native platelet-rich plasma had higher activity than that prepared from platelet-poor plasma. The liberation of platelet acid phosphatase was

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[1] Sigma Chemical Co., St. Louis, Mo.

[2] We are grateful to Dr. Waleed Maloof for selecting these patients.

TABLE I

Acid nitrophenylphosphatase activity of serum obtained from blood treated in various ways

Subject,* sex	Native platelet-poor plasma		Whole blood	Native platelet-rich plasma		Platelet contribution†
	37°	23°	23°	37°	23°	37°
	μM/ml./hr.		*μM/ml./hr.*	*μM/ml./hr.*		*μM/10⁹ platelets/hr.*
1A ♀		0.05			0.27	
2A ♀	0.23	0.19	0.34	0.96	0.45	2.37
3A ♂	0.42	0.47	0.65	0.69	0.47	1.00
4A ♂	0.46	0.51	0.55	0.82	0.59	0.91
5A ♀	0.48	0.44	0.50	0.88	0.58	0.55
2B ♀	0.42	0.36		0.91	0.55	1.02
Patients with prostate carcinoma						
6A ♂	0.65		1.50	0.76		
6B ♂	0.65	1.46	1.63	0.74	1.75	
7A ♂	0.79	2.48	2.65	1.71	2.57	

* Each number designates a different subject. Each letter indicates a different blood sample.

† $\frac{\mu M/ml./hr. \text{ in serum from platelet-rich plasma} - \mu M/ml./hr. \text{ in serum from platelet-poor plasma}}{\text{Platelet count in platelet-rich plasma in } 10^9/ml.}$.

consistently greater in the samples allowed to clot at 37° C. than in those clotted at room temperature. The amount of phosphatase contributed by the platelets was calculated by subtracting the activity of serum from platelet-poor plasma from the activity of serum from platelet-rich plasma clotted at 37° C. and dividing the difference by the platelet count in 10^9 per ml. It ranged from 0.55 to 2.37 μM nitrophenol per 10^9 platelets per hour.

The time course of phosphatase liberation is shown in Figure 1. Since retraction did not usually occur, the values on the curve were determined on serum expressed from the clot with

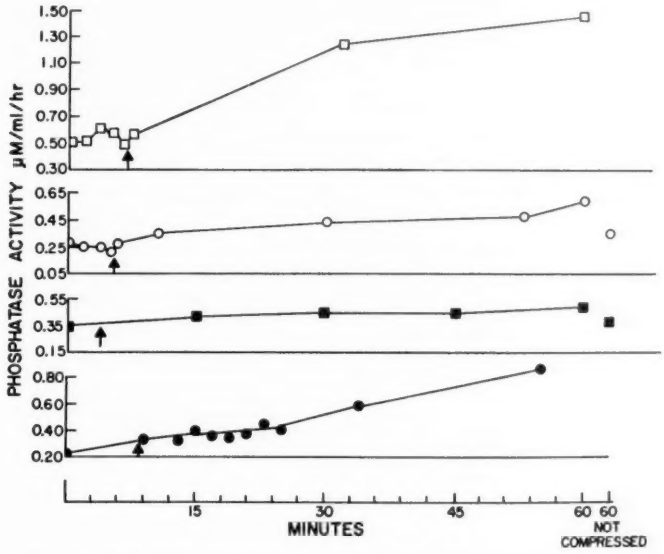

FIG. 1. ACID NITROPHENYLPHOSPHATASE ACTIVITY OF SERUM SEPARATED AT VARIOUS INTERVALS FROM NATIVE PLATELET-RICH PLASMA OF FOUR NORMAL SUBJECTS

Time of clotting is indicated by the arrow.

TABLE II

Acid nitrophenylphosphatase activity of platelet-rich and platelet-poor plasma from citrated, EDTA and oxalated blood

Subject	Time after venepuncture	Temperature	Citrated			EDTA			Oxalated		
			Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution	Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution	Platelet-rich plasma	Platelet-poor plasma*	Platelet-contribution
	hrs.	*° C.*	*μM/ml./hr.*	*μM/ml./hr.*	*μM/10⁹/hr.*	*μM/ml./hr.*	*μM/ml./hr.*	*μM/10⁹/hr.*	*μM/ml./hr.*	*μM/ml./hr.*	*μM/10⁹/hr.*
1A	1.5	?	1.12	0.22	1.10						
2A	1.5	?	0.77	0.31	1.26	0.67	0.33	0.89	1.11	0.29	2.22
3A	1.25	4	0.57	0.38	0.34	2.63	0.43	3.24	0.73	0.45	0.44
	3.5†	4				0.66	(0.43)	0.34	0.59	(0.45)	0.23
4A	1	?	0.80	0.43	1.06	1.37	(0.43)	1.83			
2B	10 min.	24	0.74	(0.39)	0.91	1.19	(0.39)	2.05			
	6†	4	0.47‡	(0.39)	0.15‡	0.45‡	(0.39)	0.15‡			
2C	10 min.	4	0.45	0.34	0.20	0.50	0.32	0.46			
	10 min.	24				0.45	0.26	0.49			

* Values in parentheses represent activity of serum from platelet-poor plasma.
† Time after first determination.
‡ Values after freezing once were 2.42, 5.36, 3.41 and 7.82 μM/ml./hr., respectively.

an applicator stick. No liberation of phosphatase was evident immediately after clotting had occurred (indicated by the arrow) but the serum activity increased progressively thereafter. In samples in which the clot was freed from the wall of the tube, allowed to retract, and removed without compression, serum phosphatase activity even at one hour showed little increase over the value obtained when the platelets were removed prior to clotting.

The activity of ordinary serum, separated by centrifugation from whole blood allowed to remain at room temperature for one hour, was usually only slightly higher (4 to 32 per cent) than that of serum from platelet-poor plasma (Table I). Activity was slightly or not at all increased by shaking the blood with glass beads during clotting.

The nitrophenylphosphatase activities of platelet-poor plasma from citrated, EDTA or oxalated plasma (Table II) showed no significant differences from each other or from serum prepared from native platelet-poor plasma from the same subject (Table I). Addition of citrate or EDTA to serum had no effect on acid nitrophenylphosphatase activity in two experiments;

TABLE III

Acid nitrophenylphosphatase activity (μM/10⁹ platelets/hr.) of washed platelets treated in various ways

Subject	0.9% NaCl, unfrozen	0.9% NaCl, frozen	0.45% NaCl, frozen		Platelet-poor plasma,* 37° C.	Viscous metamorphosis	
			Supernatant	Sediment		Supernatant	Sediment
1A	3.18		0.42	4.50		0.28	2.29
2A	1.86		2.59	6.00		0.10	1.50
3A	1.52		1.06	5.40	1.30	0.06	0.86
4A	6.54		3.90	8.40	1.00†	0.46‡	1.98‡
5A	1.53	9.31	1.58	5.86			
2B	5.12	14.40	2.48	7.63			
2C	2.48	9.58		7.50§			1.47§
2D	3.66	12.90					
Patients with prostate carcinoma							
6B	5.09	10.30	1.98	6.18		0.28	2.28
7A	5.63		5.38	9.88			
7B		3.22					

* Activity of serum from platelet-poor plasma subtracted from that of serum obtained after adding platelets, then divided by number of platelets.
† 1.00 when incubated at room temperature.
‡ Values were 0.40 and 2.26, respectively, when incubated at 37° C. rather than at room temperature.
§ Activity of entire sample, not separated into supernatant and sediment.

oxalate decreased the activity 18 per cent in a normal male subject but had no effect in a female subject. None of the anticoagulants affected the color developed by nitrophenol itself. The activity of platelet-rich plasma was always higher than that of platelet-poor plasma (Table II) but it varied in an unpredictable manner. For example, in Samples 3A and 2B, EDTA platelet-rich plasma had much more activity than citrated platelet-rich plasma, but this high activity disappeared after three to six hours at 4° C. However, in another experiment, 2C, on one of the same donors, this difference between citrated and EDTA samples was not evident even when the determinations were made very promptly on chilled blood. Because of this variability, the calculated activity per 10^9 platelets in platelet-rich plasma was sometimes lower and sometimes higher than it was in serum from native platelet-rich plasma.

Even after 30 minute incubation of platelet-rich plasma in the buffered substrate, the platelets appeared morphologically intact, with processes but no balloons or vesicles. Thus, unless some of the platelet enzyme had leaked out of the platelets despite their morphologic integrity, unruptured, unwashed platelets can hydrolyze nitrophenylphosphate. When platelet-rich plasma was frozen and thawed once, the activity was higher than that of an unfrozen sample (Table II, footnote).

Acid nitrophenylphosphatase activity was determined on washed saline-suspended platelets treated in various ways. The results are shown in Table III. Suspensions of washed unfrozen platelets had higher acid phosphatase activity than suspensions of platelets which had not been separated or washed (*i.e.*, platelets in platelet-rich plasma), and an increase of several-fold was observed after freezing and thawing the saline suspension once. The platelets were separated into supernatant and sediment by centrifuging for five minutes at 2,000 rpm after they had been frozen and thawed in 0.45 per cent saline. The sediment was resuspended in the original volume of saline. The activity of the supernatant and sediment was somewhat less than that of platelets frozen in isotonic saline, whether it was determined by adding the values for supernatant and sediment or by measuring the activity without separating the two fractions (Experiment 2D). The activity of the supernatant was between 9 and 32 per cent of the total activity and was approximately equal to the activity contributed by the platelets to serum from platelet-rich plasma.

Release of acid phosphatase activity from washed platelets during clotting was studied by adding them to native platelet-poor plasma which was allowed to incubate for 60 minutes at 37° C. before removing the clot and entrapped platelets. The activity liberated was almost identical with that liberated from unwashed platelets during the clotting of native platelet-rich plasma (cf. Table I). In other words, washing the platelets did not affect the amount of phosphatase liberated during clotting at 37° C. However, washing did facilitate phosphatase liberation at room temperature; when washed platelets were used, the same amount of phosphatase was liberated whether clotting took place at room temperature or at 37° C., whereas a much smaller amount was liberated from native platelet-rich plasma when it was clotted at room temperature rather than at 37° C.

Studies were also carried out on platelets which had undergone viscous metamorphosis (14) (Table III). To five volumes of platelets suspended in saline were added three volumes of tris-maleate buffer (pH 7.4) and two volumes of human thrombin (about 5 units per ml.) (15). After a few minutes at room temperature with occasional agitation, the platelets clumped, and soon thereafter, the platelets in the clumps fused together. After 60 minutes, the samples were separated into supernatant and sediment and the sediment was resuspended in saline. The activity of the supernatant was much less than either the activity of the supernatant obtained after freezing the platelets in hypotonic saline or the activity contributed to the serum by the platelets during clotting. The activity of the sediment was much less than that of frozen platelets, and often less than that of unfrozen washed platelets, perhaps because the platelets in the resuspended sediment were clumped.

Two patients with metastatic carcinoma of the prostate gland were studied who had elevated acid phosphatase values as determined on ordinary serum (Table I). The levels in serum

from platelet-poor plasma maintained at room temperature were above the maximum level which we observed in normal individuals (0.51 μM per ml. per hour). Thus, the prostate enzyme is not bound to platelets but is presumably in the plasma. The values for serum from platelet-poor plasma incubated at 37° C. were lower than those on serum from the same plasma incubated at room temperature, demonstrating the well-known heat lability of the prostate enzyme in serum (16, 17). Values for serum from platelet-rich plasma incubated at 23° C. were only slightly higher than those for serum from platelet-poor plasma, since liberation of the enzyme from the platelets is minimal at this temperature. Liberation of the platelet activity at 37° C. could not be quantified because of the destruction of the prostate enzyme at this temperature. The activity of acid phosphatase in washed whole platelets from two patients with elevated prostatic serum phosphatase levels was not abnormal (Table III).

Studies with inhibitors were carried out on frozen saline-suspended platelets of normal Subject 2 and Patient 6B with carcinoma of the prostate gland. L-Tartrate (18) produced 14 and 7 per cent inhibition, and formaldehyde (18) caused 81 and 76 per cent inhibition, respectively. Magnesium (0.01 M) inhibited the normal platelets 14 per cent.

DISCUSSION

Preliminary studies (9) indicated that virtually no activity against β-glycerophosphate was found in serum prepared from platelet-poor plasma, whereas values up to 0.49 Bodansky units (mg. P per 100 ml. per hour) were found in serum from platelet-rich plasma. These data suggested that the platelets are the major source of normal serum acid glycerophosphatase activity. When p-nitrophenylphosphate was used, serum from native platelet-poor plasma had some activity, indicating that the platelets were not the only source of serum activity against this substrate. However, greater activity was always observed in serum from platelet-rich plasma, especially after incubation for one hour at 37° C. In such serum, between 39 and 76 per cent of the activity was derived from the platelets. In ordinary serum, separated from whole blood centrifuged after remaining for one hour at room temperature, less than 32 per cent of the activity was attributable to the platelets.

Acid glycerophosphatase activity has been previously described in rabbit (1), horse (2), calf (3) and human (4, 7) platelets. Platelet acid phosphatase was unaltered during menstruation (4), but reduced in subjects with hepatic cirrhosis (5) or receiving dicumarol (6). Our results and those of Morita and Asada (8) indicate that human platelets are able to hydrolyze nitrophenylphosphate as well as glycerophosphate. The range of values for both frozen and unfrozen platelets of normal subjects is quite wide.

Platelet acid nitrophenylphosphatase is inhibited to a considerable degree by formaldehyde, but very little by L-tartrate or magnesium. In these respects, it behaves like the enzyme in normal serum (18, 19). Others have shown that L-tartrate inhibits acid phosphatase from prostate, liver and spleen, but not the normal serum enzyme (18). Formaldehyde partially inhibits the enzyme in serum and in several tissues, but completely inhibits the enzymes in red blood cells (18). Magnesium has little effect on acid phosphatase from most sources but may activate one of the enzymes in the red cells (19).

Investigation of factors controlling liberation of platelet acid phosphatase and perhaps other platelet enzymes (9) may shed light upon platelet function. Less than 25 per cent of the total platelet nitrophenylphosphatase activity is liberated into the serum when native platelet-rich plasma clots at 37° C. and the clot is compressed one hour later. Over two-thirds of the platelet activity against nitrophenylphosphate is insoluble and remains in the sediment after freezing the platelets in a hypotonic medium. The activity liberated during clotting is equal to between 23 and 94 per cent of the activity of the soluble platelet fraction. Liberation of enzyme from platelets occurs progressively during the hour following clotting. Both serotonin (20, 21) and potassium (22) are also liberated from the platelets during clotting, but their release occurs more rapidly than that of acid phosphatase. Although thrombin liberates all of the serotonin from washed platelets suspended in buffer (21), less phosphatase activity is liberated under these conditions than by clotting or freezing. This suggests that

viscous metamorphosis produced in this manner does not fully reproduce the platelet changes which occur after clotting of platelet-rich plasma.

Even after thorough washing, human platelets contain serotonin (21), factor V (accelerator globulin) (23) and fibrinogen (24). There is good evidence that the former two substances are taken up from the environment and bound to the platelets (23, 25), and it seems probable that platelet fibrinogen is acquired in a similar manner. Hence, one wonders whether platelet acid phosphatase has originated elsewhere and is simply carried by platelets. Since acid phosphatase can be demonstrated histologically in the cytoplasm of megakaryocytes (26), it seems unlikely that the circulating platelets take up the enzyme.

In patients with metastatic carcinoma of the prostate gland and elevated serum acid phosphatase, acid nitrophenylphosphatase activity in serum from platelet-poor plasma was above the normal level. Thus, the enzyme was presumably present in the circulating plasma. There is no evidence that the prostate enzyme becomes bound to the platelets.

SUMMARY

A significant proportion of serum enzyme activity against *p*-nitrophenylphosphate at acid pH originates from platelets and is liberated during clotting. Suspensions of intact washed platelets in saline and of unwashed platelets in platelet-rich plasma have acid nitrophenylphosphatase activity. Greater activity is observed after freezing and thawing these suspensions. Some of the factors influencing platelet liberation of acid phosphatase have been investigated. In patients with elevated serum acid phosphatase activity attributable to metastatic carcinoma of the prostate, the abnormal enzyme is presumably in the circulating plasma, since the activity of serum prepared from platelet-poor plasma is elevated, and since platelet acid phosphatase activity is normal.

REFERENCES

1. Chevillard, L. Sur les phosphomonoestérases de la moelle du fémur, des ganglions lymphatiques, des leucocytes d'exsudats chez le cobaye, et des plaquettes sanguines chez le lapin. C. R. Soc. Biol. (Paris) **1945**, **139**, 249.
2. Wagner, R., and Yourke, A. Alkaline and acid phosphatases in white blood cells and blood platelets. Arch. Biochem. 1955, **54**, 174.
3. Mizuno, N. S., Sautter, J. H., and Schultze, M. O. Enzyme and nucleic acid content of thrombocytes from normal and thrombocytopenic calves. Proc. Soc. exp. Biol. (N. Y.) 1958, **98**, 42.
4. Salvidio, E. Biochemical aspects of blood platelets (peptidase, nucleotidase, acid and alkaline phosphatase activity in normal subjects, during menstruation and in some hemorrhagic disorders). Acta. haemat. (Basel) 1954, **11**, 301.
5. Pedrazzini, A., and Salvidio, E. Enzymveränderungen der Blutplättchen bei den hypoprothrombinämischen Morgagni-Laennec'schen Lebercirrhosen. Acta haemat. (Basel) 1957, **18**, 42.
6. Salvidio, E., Pannacciulli, I., and Bianchini, E. The action of coumarin anticoagulants and of vitamin K_1 on some enzymes of human blood platelets *in* Fünfter Kongress der Europäischen Gesellschaft für Hämatologie (Freiburg, 1955), H. Begemann, Ed. Berlin, Springer-Verlag, 1956, p. 499.
7. Gude, W. D., Upton, A. C., and Odell, T. T., Jr. Blood platelets of human and rat. A cytochemical study. Lab. Invest. 1956, **5**, 348.
8. Morita, H., and Asada, T. Étude morphologique des plaquettes sanguines de l'homme. Sang 1957, **28**, 827.
9. Zucker, M. B., and Borrelli, J. A survey of some platelet enzymes and functions. The platelets as the source of normal serum acid glycerophosphatase. Ann. N. Y. Acad. Sci. 1958, **75**, 203.
10. Bessey, O. A., Lowry, O. H., and Brock, M. J. Method for rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. biol. Chem. 1946, **164**, 321.
11. Andersch, M. A., and Szczypinski, A. J. Use of *p*-nitrophenylphosphate as the substrate in determination of serum acid phosphatase. Amer. J. clin. Path. 1947, **17**, 571.
12. Serum acid and alkaline phosphatase and "prostatic" acid phosphatase using Sigma 104 Phosphatase Substrate. Technical Bulletin 104, Sigma Chemical Co., St. Louis, Mo., March, 1958.
13. Brecher, G., Schneiderman, M., and Cronkite, E. P. The reproducibility and constancy of the platelet count. Amer. J. clin. Path. 1953, **23**, 15.
14. Zucker, M. B., and Borrelli, J. Platelet viscous metamorphosis and clot retraction. Fed. Proc. 1957, **16**, 140.
15. Biggs, R., and Macfarlane, R. G. Human Blood Coagulation and Its Disorders, 2nd ed. Oxford, Blackwell, 1957.
16. Herbert, F. K. Estimation of prostatic phosphatase in serum and its use in diagnosis of prostatic carcinoma. Quart. J. Med. 1946, **15**, 221.
17. Woodard, H. Q. A note on the inactivation by heat of acid glycerophosphatase in alkaline solution. J. Urol. (Baltimore) 1951, **65**, 688.

18. Abul-Fadl, M. A. M., and King, E. J. Properties of the acid phosphatases of erythrocytes and of the human prostate gland. Biochem. J. 1949, **45**, 51.
19. Walker, B. S., Lemon, H. M., Davison, M. M., and Schwartz, M. K. Acid phosphatases, a review. Amer. J. clin. Path. 1954, **24**, 807.
20. Zucker, M. B., and Borrelli, J. Quantity, assay and release of serotonin in human platelets. J. appl. Physiol. 1955, **7**, 425.
21. Zucker, M. B., and Borrelli, J. Relationship of some blood clotting factors to serotonin release from washed platelets. J. appl. Physiol. 1955, **7**, 432.
22. Hartmann, R. C., Auditore, J. V., and Jackson, D. P. Studies on thrombocytosis. I. Hyperkalemia due to release of potassium from platelets during coagulation. J. clin. Invest. 1958, **37**, 699.
23. Hjort, P., Rapaport, S. I., and Owren, P. A. Evidence that platelet accelerator (platelet factor 1) is adsorbed plasma proaccelerin. Blood 1955, **10**, 1139.
24. Seligmann, M., Goudemand, B., Janin, A., Bernard, J., and Grabar, P. Études immunochimiques sur la présence de fibrinogène dans les extraits de plaquettes humaines lavées et dans certains extraits leucocytaires. Rev. Hémat. 1957, **12**, 302.
25. Weissbach, H., Bogdanski, D. F., and Udenfriend, S. Binding of serotonin and other amines by blood platelets. Arch. Biochem. 1958, **73**, 492.
26. Rabinovitch, M., and Andreucci, D. A histochemical study of "acid" and "alkaline" phosphatase distribution in normal human bone marrow smears. Blood 1949, **4**, 580.

MICROELECTROPHORESIS OF BLOOD PLATELETS AND THE EFFECTS OF DEXTRAN *†

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The intravenous administration of dextran has previously been shown to produce a bleeding tendency in laboratory animals and in humans. Extensive studies concerning the hemostatic defect have yielded much useful information. Carbone, Furth, Scott and Crosby (1) found that the major abnormality consisted of a prolongation of the bleeding time. In seven of 11 patients given 1,500 to 6,500 ml. of dextran intravenously over a five day period the bleeding time was prolonged over 30 minutes. The concentration of platelets, clotting time, fibrinogen level, clot retraction time and prothrombin consumption test were not significantly affected by dextran. The prothrombin time was slightly abnormal. It appears unlikely that this finding explains the hemostatic defect because of the inconstancy and mild degree of the change. Adelson, Crosby and Roeder (2) found that smaller amounts of dextran were required to produce a prolonged bleeding time in dogs made thrombocytopenic by irradiation than in normal dogs. Comparable volumes of gelatin in the thrombocytopenic dogs did not prolong the bleeding time. Hemodilution, therefore, appears to be eliminated as a cause of the defect. These results have suggested that the platelet, the vessel wall, or both may be affected by dextran.

Since there is ample indication that the platelet is concerned with changes in the bleeding time, it seemed pertinent to procure more evidence concerning the effect of dextran on the platelet. Rothman, Adelson, Schwebel and Langdell (3) have shown that C^{14} labeled dextran is taken up by the platelet. In substantiation of this effect Ponder noted that the electrophoretic mobility of the platelet in saline appeared to be modified by dextran (4). He pointed out, however, that the electrophoretic mobility may be influenced by factors that are difficult to control or appraise. In the determination of the isoelectric point these uncontrolled factors may be eliminated or reduced significantly in their importance.

Microelectrophoresis has been used rather extensively to study the red cell and those changes in its environment that affect its surface charge (5, 6). Recently Creger, Tulley and Hansen (7) utilized this method to determine the effect of hydrocortisone on red cell-antibody union. Relatively few studies of the platelet have been carried out by microelectrophoresis. Abramson (8) found that the horse platelet migrated at a rate of 0.45 μ per second per volt per cm. in oxalated plasma. The pH was not recorded. The true mobility was determined by correcting for the electroosmotic effect at the center of the electrophoresis cell. This determination of the true mobility is accomplished more simply by observing the mobility at one of the stationary levels of the cell. Our studies include observations on the electrophoretic mobility and isoelectric point of platelets suspended in various buffer and protein solutions and the effect of dextran on these measurements.

METHOD

Blood was obtained from patients without hematologic disease and from normal humans. Multiple donors were used for the determination of each isoelectric point. Blood was drawn using nonwettable equipment. Platelet suspensions were prepared in the following manner: *1)* A silicone-coated needle was inserted into an antecubital vein after a tourniquet had been applied for no more than one minute. The first few drops of blood were discarded. *2)* Fifteen ml. of blood was then permitted to flow down the side of a silicone-coated tube containing 0.5 ml. of 5 per cent sequestrene. *3)* Thirty ml. of blood was spun at 5 to 10° C. in a refrigerated (International

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PR-2) centrifuge at 1,000 rpm for 25 minutes. *4*) The supernatant was aspirated with a plastic dropper and placed in a silicone-coated tube. *5*) This was then recentrifuged at 2,000 rpm for 25 minutes at 5 to 10° C. *6*) The plasma was aspirated and the button of platelets was resuspended in 0.912 per cent saline. *7*) The latter was centrifuged at 2,000 rpm and the supernatant aspirated and discarded. *8*) The button of platelets was washed with 2 to 3 ml. of suspending solution (chilled) and resuspended in 20 ml. of suspending solution. The suspending solution was saline, or a mixture of saline and protein, or saline and dextran. The final ionic strength was 0.156. It was assumed that the proteins in the various solutions did not contribute significantly to the ionic strength. The pH of the saline or saline dextran solution was adjusted with citrate and phosphate buffers prepared according to Clark (9). The pH values of the protein solutions were adjusted by titration with 0.01 N HCl. Hemoglobin solutions were prepared by hemolyzing the blood with distilled water and removing the stroma with toluene. Human albumin and gamma globulin solutions were supplied by courtesy of the American Red Cross.

Specific conductivity measurements were made using General Radio Company Impedance Bridge, Type No. 650A, a CDA and a CDB Cornell-Dubilier Decade Capacitor, and a Leeds & Northrup Jones Conductivity Cell, Catalog No. 4914. Hopps and Engles (10) have discussed problems related to these measurements. Viscosity measurements were made using Ostwald viscosimeters. A horizontal type Abramson microelectrophoresis apparatus[1] was used (6). This cell was set in a plastic base which in turn was fixed to the stage of a phase microscope so that the center of the cell lay under the objective lens. One of the oculars contained a grid which was calibrated and served as the distance marker. The oculars were 20× and the objective was a 20× long working distance phase lens. The cell was filled with platelet suspension by turning the stopcocks to communicate with the funnel and waste outlet. All visible air bubbles were removed. The stopcocks were turned to close the cell and circuit and the current turned on. A two way switch allowed the current to be reversed manually and automatically reversed the current every one to three minutes. The cell was calibrated to make certain it was of proper shape and dimensions. This was accomplished by focusing at the top of the cell and timing the movement of platelets in first one and then the opposite direction over the measured distance. Usually 10 to 20 readings yielded a reliable average figure. These readings were taken at several levels from the top to the bottom of the cell.

The rate of migration was then plotted against cell depth. If the cell was made of uniform dimensions, a parabolic curve formed. According to the established theories of Smoluchowski (11) and Komagata (12), if the ratio of width to depth of the cell is adequate, the mean height of this curve will intersect the parabolic curve at 20.2 per cent to 21.1 per cent of the depth from the top as well as from the bottom of the cell. These levels then represent the stationary layers where mobility measurements unaffected by electro-endosmosis may be made. All measurements were made at one of these levels. The corrected velocity in μ per second per volt per cm. is equal to

$$\frac{V_{obs}\, K_s\, q\, \eta_r}{I}$$

where V_{obs} is the observed velocity in μ per second; K_s, the specific conductivity; q, the cross-sectional area of the microelectrophoresis cell in cm.^{2}; I, the current in amperes; and η_r, the relative viscosity. A discussion of some of the technical problems associated with this technique is given by Ponder and Ponder (13).

RESULTS

Mobility measurements of platelets were made in various citrate and phosphate buffers ranging in pH from 7.5 to 3.2. The corrected velocities were then plotted against pH and connected by a solid black line as shown in Figure 1. The isoelectric point was assumed to be the intersection of the zero mobility and the curve. The isoelectric point of platelets in buffered saline solution was 3.98 (Table I).

By this technique the curve and isoelectric point of saline washed platelets were then determined in serum, 1 per cent gamma globulin, and 4 per cent gamma globulin with pH adjusted by 0.01 N HCl. The isoelectric points ranged from 4.41 to 4.59 (Table I). These isoelectric points were significantly different ($p < 0.001$) from that of platelets in citrate buffer.

The isoelectric point of platelets in 3 per cent albumin was found to be 4.18. The difference between the isoelectric points of platelets in citrate buffer and those in 3 per cent albumin was significant ($p < 0.001$).

The effect of dextran[2] was studied by allowing washed platelets to remain in 3 per cent or 6 per cent dextran for 30 minutes. These platelets were then resuspended in a solution having a final concentration of 3 per cent dextran and an identical citrate concentration and ionic strength as in the previously described experiments. The isoelectric points of platelets exposed to 3 per cent and 6 per cent dextran for 30 minutes were

[1] Built by Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.

[2] The dextran solution was supplied by courtesy of the R. K. Laros Co. It contained 6 per cent hydrolyzed fractionated dextran in 0.9 per cent sodium chloride solution for clinical use.

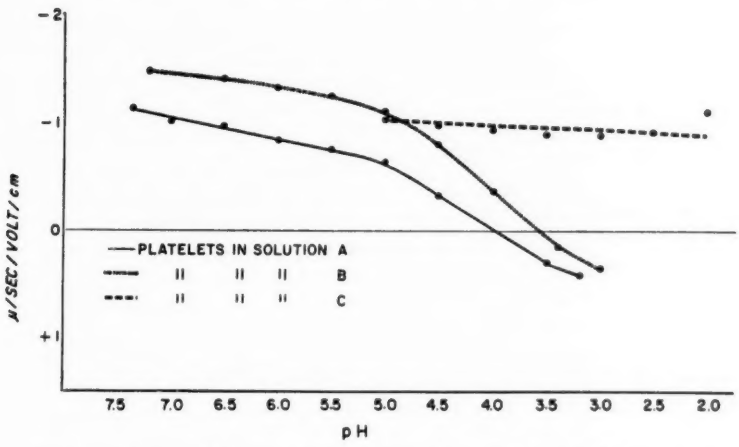

FIG. 1. CORRECTED VELOCITY CURVE OF PLATELETS IN VARIOUS SOLUTIONS AT DIFFERENT pH LEVELS

Solution A is made up of one part citrate or phosphate buffer and nine parts of saline. Solution B is made up of 6 per cent dextran in one part citrate or phosphate buffer and nine parts of saline. Solution C is made up of 0.5 mg. per cent heparin in one part citrate or phosphate buffer and nine parts of saline.

3.83 and 3.63, respectively. These differ significantly ($p < 0.001$) from the isoelectric point of platelets unexposed to dextran (Table I).

Platelets were exposed to 6 per cent dextran for 30 minutes. Serum and saline solution was added so that the final concentration of dextran was 3 per cent and of serum protein approximately 1 per cent. The isoelectric point was found to be 4.49 and was not significantly different from that obtained with platelets unexposed to dextran but suspended in serum. This clearly demonstrates that dextran does not prevent coating of the platelets by serum proteins.

Since most of the isoelectric points were found to be in a fairly narrow range it seemed desirable to explore the possibility of producing more marked changes in the isoelectric point of the platelets. The isoelectric point of platelets suspended in a 3 per cent hemoglobin solution was determined in the previously described manner and was found to be 5.27 (Table I). The difference between this and the isoelectric

TABLE I

Isoelectric points of saline washed human platelets suspended in various solutions*

Pretreatment	Suspending solution	I.E.P.†	S.D.‡	Range
	0.5 mg. % Heparin in P-C-S§	<2.18		
Exposed to 6% dextran, 30 min.	3% Dextran in P-C-S	3.63	0.10	3.48–3.80
Exposed to 3% dextran, 30 min.	3% Dextran in P-C-S	3.83	0.09	3.72–3.92
	P-C-S	3.98	0.08	3.83–4.10
3 saline washes	P-C-S	3.99	0.06	3.91–4.05
	3% Albumin in saline	4.18	0.08	4.08–4.27
	Human serum	4.41	0.16	4.13–4.58
	1% Gamma globulin in saline	4.49	0.13	4.34–4.68
Exposed to 6% dextran, 30 min.	Serum protein 1%, dextran 3%	4.49	0.08	4.38–4.62
	4% Gamma globulin in saline	4.59	0.04	4.54–4.63
	3% Hemoglobin	5.27	0.30	4.93–5.78

* Unless otherwise stated, platelet suspensions were washed once with saline.
† Isoelectric point.
‡ Standard deviation.
§ One part of citrate or phosphate buffer and nine parts of saline solution.

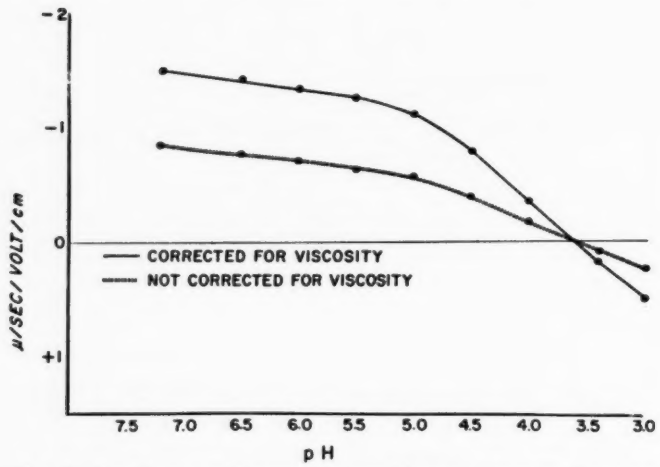

FIG. 2. THE EFFECT OF VISCOSITY ON THE MOBILITY CURVE OF PLATELETS SUSPENDED IN A SOLUTION CONTAINING 6 PER CENT DEXTRAN AND ONE PART CITRATE OR PHOSPHATE BUFFER IN NINE PARTS OF SALINE

point of platelets in 4 per cent gamma globulin was significant ($p < 0.001$). At the other end of the scale platelets suspended in 0.5 mg. per cent heparin and citrate buffer were negatively charged at a pH as low as 2.18 (Figure 1). The isoelectric point was not determined because of the instability of the velocity at these low pH values. Readings had to be made in less than two minutes or sudden changes in direction of migration would occur. Heparin was added to serum and solutions of albumin and gamma globulin. Platelets suspended in these solutions were found to have the same isoelectric point as platelets in similar protein solutions without heparin. This was true with concentrations of 2.0 mg. per cent heparin in the final solution.

It is evident from these experiments that the isoelectric point of platelets is modified by the suspending medium. The isoelectric points of platelets in the protein solutions are in the same relative order as the isoelectric points of those proteins. In no case, however, is the isoelectric point of the platelets the same as that of the protein. This may represent a partial coating of the particle which would fit Abramson's key spot hypothesis (6). It is also interesting to note that Abramson (14) found that quartz particles suspended in serum had an isoelectric point of 4.7 which is close to the isoelectric point of platelets in serum.

DISCUSSION

The theory of Helmholtz and Lamb indicates that electrophoretic mobility should be proportional to the zeta potential and inversely proportional to the viscosity of the medium, provided that the thickness of the double layer, the facility of slip and the dielectric constant remain unchanged. Ponder (4) used this relation in the form

$$\frac{U}{U_0} = \frac{v\,\eta}{v_0\,\eta_0}$$

where U was the zeta potential, v the mobility, η the viscosity, and the subscript zero referred to saline without dextran. His values of U/U_0 for platelets in 1 per cent dextran at pH 7.0 averaged 1.27, while our figure for platelets in 3 per cent dextran at pH 7.0 is 1.40.

Ponder questioned the validity of treating platelets as molecular particles with regard to the effect of viscosity on flow. The determination of the isoelectric point eliminates the problem of how to treat the effect of viscosity. In our experiments the isoelectric point was observed to be essentially unchanged whether or not the points of the curve were plotted with a consideration for the effect of viscosity (Figure 2).

The platelet suspended in a protein solution migrates in an electrical field in a fashion comparable to leucocytes and inert particles. The

evidence gained from electrophoresis suggests that the platelet is partially coated by the protein in which it bathes. This is in sharp contrast to the evidence that erythrocytes suspended in a protein solution are not coated by this protein (15, 16). It appears that there may be a variance in the type or degree of such coating between leucocytes and platelets but there is still an inadequate amount of information about the former to warrant any final conclusion.

Abramson, Gorin and Ponder (17) concluded that platelets suspended in serum are coated by serum albumin A. They arrived at this conclusion on the basis of similar mobilities among quartz particles, leucocytes and platelets in serum and the evidence by Moyer and Moyer (18) that quartz particles are coated by serum albumin A. In view of the isoelectric point differences between platelets in albumin and in serum it appears that platelets suspended in serum must be at least partially coated by globulin.

The effect of dextran on platelet mobility is interesting in that dextran is essentially nonionic. A possible reason for the effect of dextran on platelet mobility is that the dextran might conjugate with some of the proteins on the cell surface and by so doing may change the orientation of the proteins. Support for this view is found in the observation of Rozenfel'd and Plyshevskaya (19) that dextran does conjugate with protein.

The fact that dextran does not influence the isoelectric point of platelets suspended in serum does not militate against a significant influence of dextran on the surface of the platelet. It should be noted that heparin had a pronounced effect on the platelet mobility when the platelets were suspended in saline but that even in extremely high concentrations it did not affect the mobility of platelets in protein solutions. These latter results are in the face of Zucker's (20) observation that high doses of heparin increase the mesenteric bleeding time of rats and prevent the formation of an adequate platelet thrombus. Abramson's "key spot" hypothesis again may come into play as an explanation of the apparent dichotomy of results. He showed that sheep erythrocytes when sensitized by the addition of small amounts of rabbit amboceptor did not have their electrophoretic mobility altered and still could be hemolyzed by the addition of complement.

SUMMARY AND CONCLUSIONS

1. The isoelectric point of saline washed human platelets suspended in citrate buffer is 4.0.

2. When platelets are suspended in a solution containing human albumin, serum, gamma globulin or hemoglobin, the platelets appear to be partially coated by the protein in the surrounding medium, as evidenced by a change in the isoelectric point toward that of the protein.

3. Platelets suspended in serum have an isoelectric point significantly different from platelets suspended in albumin solutions alone but not significantly different from platelets suspended in a solution containing gamma globulin.

4. Heparin produces a marked increase in the negative charge of platelets in citrate buffer.

5. Dextran causes an alteration in the mobility and a significant lowering of the isoelectric point of platelets in citrate buffer.

6. Our observations support the view that dextran affects the platelet surface. It is possible but unproven that this change prevents the platelets from forming a platelet thrombus in dextran treated animals.

ACKNOWLEDGMENT

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REFERENCES

1. Carbone, J. V., Furth, F. W., Scott, R., Jr., and Crosby, W. H. An hemostatic defect associated with dextran infusion. Proc. Soc. exp. Biol. (N. Y.) 1954, **85**, 101.
2. Adelson, E., Crosby, W. H., and Roeder, W. H. Further studies of a hemostatic defect caused by intravenous dextran. J. Lab. clin. Med. 1955, **45**, 441.
3. Rothman, S., Adelson, E., Schwebel, A., and Langdell, R. D. Adsorption of carbon-14 dextran to human blood platelets and red blood cells, in vitro. Vox Sang. (Basel) 1957, **2**, 104.
4. Ponder, E. Revêtement des plaquettes humaines et des globules rouges par les dextranes. Rev. Hémat. 1957, **12**, 11.
5. Abramson, H. A. The cataphoretic velocity of mammalian red blood cells. J. gen. Physiol. 1929, **12**, 711.

6. Abramson, H. A., Moyer, L. S., and Gorin, M. H. Electrophoresis of Proteins and the Chemistry of Cell Surfaces. New York, Rheinhold Publishing Corp., 1942.
7. Creger, W. P., Tulley, E. H., and Hansen, D. G. A note on microelectrophoresis of red cells. Stanf. med. Bull. 1955, **13**, 16.
8. Abramson, H. A. The electrophoresis of the blood platelets of the horse with reference to their origin and to thrombus formation. J. exp. Med. 1928, **47**, 677.
9. Clark, W. M. The Determination of Hydrogen Ions, 3rd ed. Baltimore, Williams and Wilkins Co., 1928.
10. Hopps, H. C., and Engles, C. F. Some technical considerations in electrophoretic studies of cells and other small particles. Lab. Invest. 1957, **6**, 77.
11. Smoluchowski, M. *in* Graetz. Handbuch der Elektrizitat un des Magnetismus. Leipzig, Barth. 1921, **2**, 366. Cited by Abramson, Moyer, and Gorin (6), ch. 3.
12. Komagata, S. Researches Electrotech. Lab. Tokyo, No. 348, 1933. Cited by Abramson, Moyer, and Gorin (6), ch. 3.
13. Ponder, E., and Ponder, R. V. Electrophoretic mobility of red cells and their ghosts as observed with improved apparatus. J. exp. Biol. 1955, **32**, 175.
14. Abramson, H. A. Elektrokinetic phenomena. I. The adsorption of serum proteins by quartz and paraffin oil. J. gen. Physiol. 1929, **13**, 169.
15. Abramson, H. A. Electrokinetic Phenomena and Their Application to Biology and Medicine. New York, Chemical Catalog Co., 1934.
16. Abramson, H. A., Furchgott, R. F., and Ponder, E. The electrophoretic mobility of rabbit erythrocytes and ghosts. J. gen. Physiol. 1939, **22**, 545.
17. Abramson, H. A., Gorin, M. H., and Ponder, E. Electrophoresis and the chemistry of cell surfaces. Cold Spr. Harb. Symp. quant. Biol. 1940, **8**, 72.
18. Moyer, L. S., and Moyer, E. Z. Electrokinetic aspects of surface chemistry. VII. Electrophoretic behavior of microscopic particles in the presence of horse, human, or rabbit serum. J. biol. Chem. 1940, **132**, 373.
19. Rozenfel'd, E. L., and Plyshevskaya, E. G. Some peculiarities of dextran and its interaction with blood proteins. Doklady Akad. Nauk S.S.S.R. 1954, **95**, 333 *in* Chem. Abstr. 1954, **48**, 8283.
20. Zucker, M. B. Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal, thrombocytopenic, heparinized, and hypoprothrombinemic rats. Amer. J. Physiol. 1947, **148**, 275.

TRANSFER OF IRON FROM SERUM IRON-BINDING PROTEIN TO HUMAN RETICULOCYTES *

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The metabolism of iron involves a series of compounds which restrict iron to undissociated forms. Upon absorption by gut mucosal cells, iron is enveloped within ferritin molecules (1, 2). In the presence of reducing agents, ferritin iron then is released to the surfaces of ferritin molecules (3) to become bound by a specialized transport protein of the plasma.[1] This iron-binding protein (IBP) is a β_1 globulin by electrophoretic analysis but resembles albumin in chemical properties (5, 8). Each molecule of IBP from human plasma or from the plasma of several other mammalian species studied, in common with the IBP of egg white, "conalbumin," binds two iron atoms to form a colored complex (5, 9–12). Iron bound by these proteins is in the ferric state (5, 8) and is tightly chelated in complexes which apparently contain three phenolic groups and one bicarbonate ion (7, 11, 12). At a physiologic pH and bicarbonate concentration the iron-IBP complex is extremely stable. In some manner, however, iron is transferred from IBP to immature red cells for incorporation into heme. Although it is well established that immature red cells, either as intact cells or as hemolysates, can utilize ionic iron for heme synthesis (13–20), little is known of the mechanism releasing iron from plasma IBP for cellular utilization. It is often assumed that this mechanism for transferring iron from IBP to immature red cells includes transition through marrow iron storage sites. Recently, indeed, Bessis and Breton-Gorius (21, 22) have interpreted their electron-microscopic studies of immature red cells to indicate that erythroblasts may obtain iron for hemoglobin synthesis by removing iron particles from contiguous macrophages. The physical disadvantages of such a system as compared to a soluble transfer system are great, however, and the observation by Walsh and co-workers (15) that intact immature red cells (human reticulocytes) are capable of taking up iron bound to plasma *in vitro* suggests that normally iron is transferred directly from IBP to immature red cells.

The interaction between IBP and immature red cells represents a physiologic transfer system peculiarly susceptible to study *in vitro*. Observations characterizing this interaction, partially described in a preliminary report (23), are presented below.

MATERIALS

Iron-binding protein of a high degree of purity was prepared as described elsewhere by Inman (24), using as a starting material Fraction IV-7, obtained by standard ethanol fractionation procedures and saturated with iron.[2] After recrystallization six times this material was analyzed by electrophoresis and found to contain 97.6 per cent iron-binding protein. Its molecular weight was calculated from data obtained by ultracentrifugation and by diffusion to be 87,000 (6).

Iron-saturated conalbumin, prepared from hen's eggs, was generously supplied by Dr. Robert C. Warner.[3] The

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[1] Although several metals, including Cu^{++}, Zn^{++} (4, 5), Co^{+++} and Mn^{+++} (6) may combine with this protein, its sole known physiologic function is to transport iron through plasma. Thus the terms transferrin (4), siderophyllin (7), and iron-binding protein, of which the last will be employed in this report, are perhaps preferable to less specific terms such as metal-binding protein or metal-combining globulin.

[2] In this method the pH is not reduced below 5.2. Below a pH of 5, this protein is at least partially denatured. Partial denaturation of IBP during its preparation presumably accounts for differences reported (25) in the turnover of iron bound to unaltered plasma IBP as compared to iron bound to certain commercial fractions of IBP.

[3] Department of Biochemistry, New York University College of Medicine, New York, N. Y.

iron in this material was removed by dialysis against a 0.4 per cent solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA) [4] in physiologic saline adjusted to pH 5.6, and EDTA was subsequently removed by repeated dialysis against iron-free saline buffered at pH 7.4. Throughout these experiments Fe^{59} [5] was derived from dilute acid solutions of radioactive ferric chloride to which four parts of stable carrier iron were usually added. Studies of cobalt utilization by red cells were carried out with $Co^{60}Cl_2$.[5]

Whole heparinized blood samples rich in reticulocytes were generally obtained from patients either with pernicious anemia during therapeutic response or with hemolytic anemias. In certain instances the proportion of reticulocytes was increased by centrifuging and withdrawing the top portion of packed red cells. Finally, saline suspensions of red cells were usually prepared by washing the specimens of heparinized peripheral blood three times by centrifugation in approximately 20 volumes of iron-free physiologic saline. For the sake of brevity such reticulocyte-rich red cell suspensions are referred to below in many places simply as "reticulocytes."

METHODS

Hematocrit and hemoglobin values of the washed red cell preparations were determined by standard methods (26); reticulocytes were enumerated by the dry method (26) after resuspension of a drop of washed cells in two drops of plasma.

Complexing of iron and IBP was produced by slowly adding $Fe^{59}Cl_3$ to solutions of IBP in iron-free physiologic saline containing 10^{-2} M $NaHCO_3$. This mixture was allowed to react for one hour at room temperature before addition to red cells, although color due to the formation of the Fe-IBP complex regularly reached a maximum within a few minutes of admixture.

Cobalt and manganese also form stable colored complexes with IBP and, as with iron, the trivalent state is required for this reaction (6). Unlike iron, which autooxidizes readily to the ferric state under physiologic conditions, cobalt requires the presence of an oxidant in order to remain in the cobaltic form. Thus one-tenth volume of 0.1 M H_2O_2 was added to the reaction mixture containing $Co^{60}Cl_2$, $NaHCO_3$ and IBP.

In the majority of experiments a 30 μM concentration of IBP was employed and equal volumes of 20 or 30 μM concentrations of metal were added, resulting in one-third and one-half saturation, respectively, of the iron-binding capacity of IBP. Actual determinations of the iron-binding capacity of purified IBP, plasma or serum were made by the method of Rath and Finch (27), and iron concentrations were measured by the method of Kitzes, Elvehjem and Schuette (28).

Following addition of washed red cells to the Fe^{59}- or Co^{60}-containing test mixtures of the type described above, the mixed suspensions were incubated at 37° C. with agitation. At the end of the incubation period the red cells were washed with physiologic saline five times. The packed washed red cells were then diluted with physiologic saline to a given volume in volumetric flasks, from which 3 ml. samples were withdrawn for determination of whole red cell radioactivity in a well-type scintillation counter. The red cell radioactivity was compared to that of the standards, and the red cell uptake of metal was generally expressed as micrograms of metal per milliliters of cells.

The fraction of radioactivity not present in the red cell membranes was estimated in most experiments by centrifuging 5 ml. of the final cell suspension and hemolyzing the sedimented cells with 3 ml. of distilled water; 0.7 ml. of 5 per cent NaCl was then added, and the suspension was made up to volume in a 5 ml. volumetric flask with 0.85 per cent saline. The hemolyzed suspension was then centrifuged clear of cell membranes at approximately 1,000 G for 15 minutes and the supernatant solution was assayed for radioactivity. The localization of Fe^{59} in red cell fractions was more precisely defined in certain instances by differential centrifugation of hemolysates with an ultracentrifuge.[6] The incorporation of Fe^{59} into heme was determined in certain experiments by measuring the radioactivity of heme extracted from the red cells by the method of Fischer (29).

In order to estimate simply and simultaneously the distribution of Fe^{59} between hemoglobin, IBP and the red cell membranes, filter paper electrophoresis and autoradiography were employed. In this procedure, the washed reticulocytes were suspended in equal volumes of saline and hemolyzed by repeated freezing and thawing. At pH 6.35, midway between the isoelectric points of IBP (5.9) and hemoglobin (6.8), these two proteins migrated oppositely, whereas the particulate matter of the cells remained precipitated at the origin. The proportion of radioactivity traveling with the hemoglobin spot was determined by planimetric analysis and quantitated by comparison with autoradiographs of various dilutions of the Fe^{59} standard. In intact cell systems, good conformity appeared between measurement of hemoglobin incorporation of Fe^{59} by this method, and heme incorporation of Fe^{59} by the method of Fischer. By the autoradiograph method, over 80 per cent of the radioactivity of supernatant solutions derived from water hemolysates of Fe^{59}-labeled reticulocytes, prepared as described above, resided in the hemoglobin spot.

RESULTS

The uptake of $Fe^{59}Cl_3$ from saline by mature and immature red cells

Fe^{59} from a saline solution of $Fe^{59}Cl_3$ was bound to, or adsorbed onto, both mature and immature red cells (Figure 1). The uptake of radioactive Fe^{+++} by both mature and immature

[4] "Sequestrene," three times recrystallized, Alrose Chemical Company, Providence, R. I.

[5] Abbott Laboratories, North Chicago, Ill.

[6] Spinco Model L Preparative Ultracentrifuge.

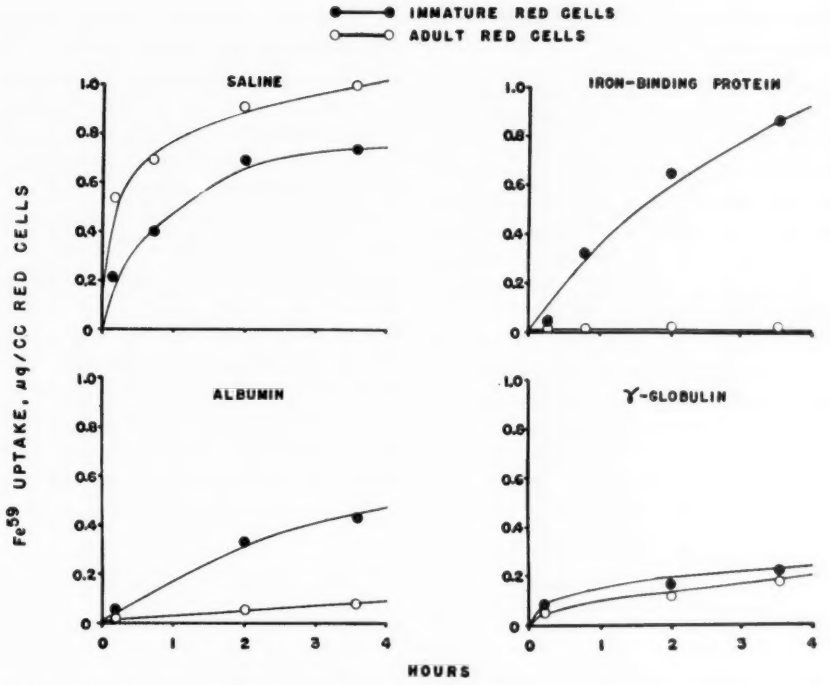

FIG. 1. THE INFLUENCE OF PROTEIN BINDING ON THE UPTAKE OF IRON BY IMMATURE AND MATURE RED CELLS

$Fe^{59}Cl_3$ was added to saline, albumin, iron-binding protein and γ-globulin solutions. After one hour at room temperature these mixtures were incubated with a washed suspension of red cells containing 31.5 per cent reticulocytes from a patient with acquired hemolytic anemia (immature red cells) and with a washed suspension of normal adult red cells, only 0.3 per cent of which were reticulocytes.

The uptake of Fe^{59} by the washed cells after various periods of times was then measured as described under "Methods." Note the striking selectivity with which immature red cells took up Fe^{59} from the iron-binding protein.

red cells was initially very rapid, and gradually diminished thereafter. Approximately 90 per cent of the Fe^{59} adsorbed by these red cells remained in the membrane fraction after water hemolysis and centrifugation at 1,000 G; and when the uptake of Fe^{59+++} exceeded about 5 μg. per ml. red cells, red cell agglutination ensued, a finding reported in more detail elsewhere (30). Siderocyte granules were not observed in such iron-coated red cells.

Although the uptake of Fe^{+++} from saline by the reticulocytes of patients with various diseases was usually similar to the uptake by mature red cells obtained from these same patients, the Fe^{+++} uptake of reticulocyte-poor suspensions of red cells from patients with acquired anemia and a positive Coombs test was consistently smaller than that of reticulocyte-rich suspensions prepared from the same blood specimens (Figure 2) or of suspensions of normal mature red cells (Figure 1). In pursuance of this finding, the effect of red cell sensitization with antibodies upon Fe^{+++} uptake was assessed by comparing the uptake of Fe^{59} from 30 μM $Fe^{59}Cl_3$ in one hour by washed normal red cells and by normal cells which had been sensitized before washing with serum containing incomplete anti-D. The iron uptake of anti-D sensitized red cells was 2.18 μg. per ml. red cells as compared to 3.43 μg. of iron per ml. of unsensitized red cells.

The uptake of Fe^{59} bound to IBP by mature and immature red cells

Iron bound to IBP (as 30 μM, half-saturated Fe^{59} IBP) was not taken up by mature red cells despite incubation at 37° C. for as long as 24 hours. However, with red cell suspensions rich in reticulocytes an appreciable transfer of iron from IBP to the cells occurred, with a slowly diminishing rate of uptake over the course of several hours of incubation (Figure 1). By contrast, iron bound to human albumin (Cohn Fraction V), in a concentration equimolar to that of the IBP studied, was transferred to some extent to mature red cells. Although iron was preferentially taken up from albumin by reticulocyte-rich cell suspensions, the uptake by these cells was less than was the case with IBP (Figure 1). Thus, with iron bound to albumin there was less discrimination between mature and immature cells than with iron bound to IBP. When iron was bound to an equimolar concentration of human gamma globulin (Cohn Fraction II-1,2) the iron uptake by adult red cells was still greater (Figure 1) but with no discrimination between adult and immature red cells.

In contrast to free iron, iron bound to IBP (Fe^{59} IBP) was taken up by a suspension of red cells in direct proportion to its concentration of reticulocytes (Figure 2). By extrapolation of data, assuming that iron was taken up only by reticulocytes, the quantity of iron transferred in two hours from IBP to the red cells of various patients ranged from 0.5 to 4.5 μg. per ml. reticulocytes. In general the amount transferred correlated with the apparent morphologic immaturity of the reticulocytes. Also in contrast

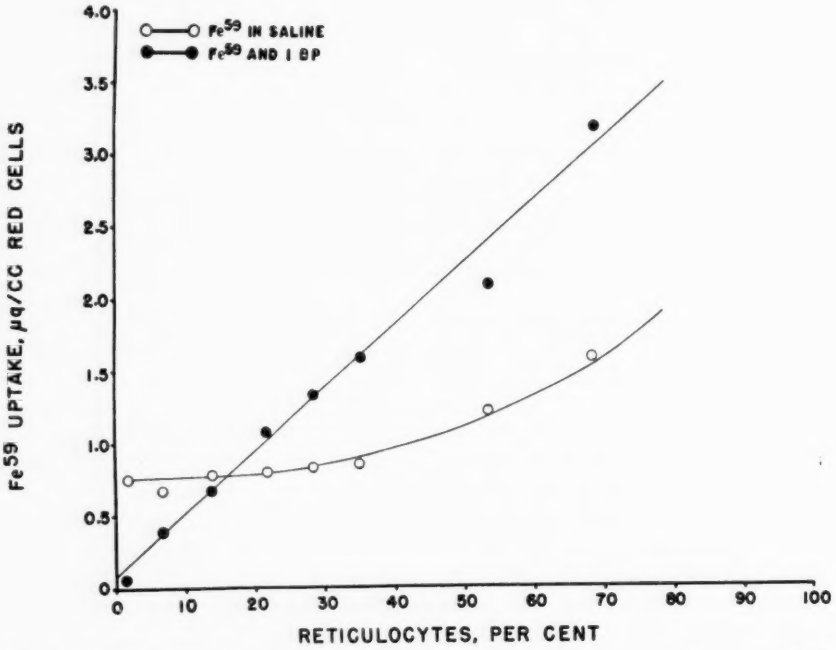

FIG. 2. THE RELATION BETWEEN RETICULOCYTE CONCENTRATION AND THE UPTAKE OF Fe^{59} FROM $Fe^{59}Cl_3$ AND FROM Fe^{59} IBP

Cell suspensions containing various reticulocyte concentrations were obtained by combining the top (reticulocyte-rich) and bottom (reticulocyte-poor) fractions of the centrifuged red cells of patients with acquired hemolytic anemia in different proportions. The uptake of Fe^{59} from Fe^{59} IBP is seen to occur in direct proportion to the reticulocyte concentration. As noted in the text, the somewhat greater uptake of Fe^{59} from $Fe^{59}Cl_3$ by reticulocytes than by adult cells was peculiar to patients with acquired hemolytic anemia.

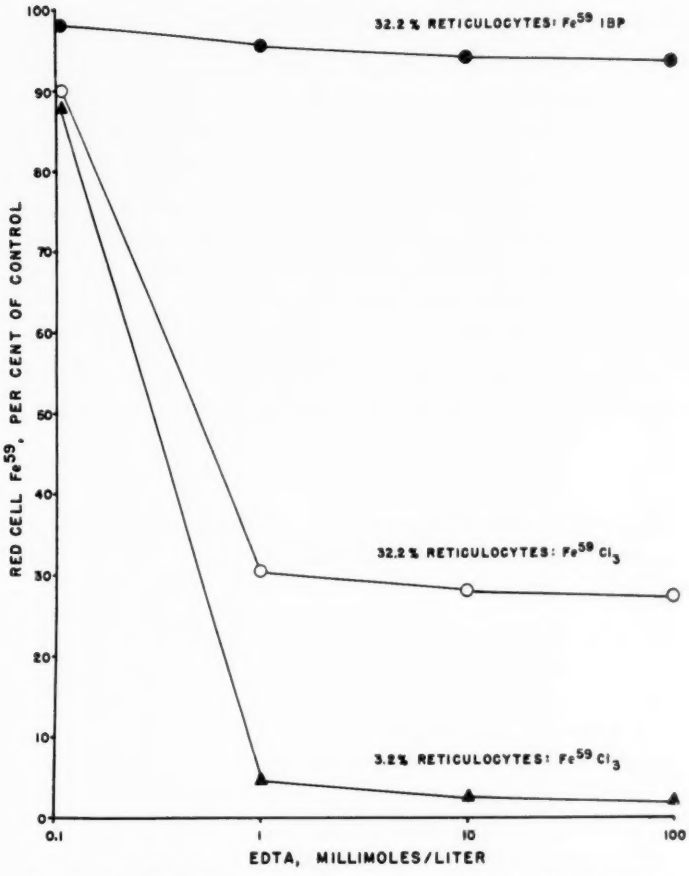

FIG. 3. THE ELUTABILITY FROM RED CELLS OF Fe^{59} TAKEN UP *IN VITRO*

As indicated by the solid circles, very little of the Fe^{59} taken up from Fe^{59} IBP by a reticulocyte-rich suspension of red cells could be eluted by subsequent exposure to high concentrations of EDTA, albeit over half of the Fe^{59} was in the cell membranes. Fe^{59} taken up from $Fe^{59}Cl_3$ by a reticulocyte-poor suspension of red cells from the same patient was almost entirely eluted by EDTA (solid triangles). Fe^{59} taken up from $Fe^{59}Cl_3$ by the reticulocyte-rich cell suspension was partially eluted, but almost 30 per cent of the Fe^{59} resisted elution by high concentrations of EDTA (empty circles), although less than 12 per cent of the Fe^{59} had been incorporated into hemoglobin.

to the 10 per cent of Fe^{59} derived from $Fe^{59}Cl_3$ in saline, from 40 to 90 per cent of red cell Fe^{59} derived from Fe^{59} IBP was recoverable in the supernatant solution of water hemolysates of reticulocyte-rich blood, spun at about 1,000 G. Thus from 10 to 60 per cent of the Fe^{59} was associated with the reticulocyte membranes. In a representative experiment, 49.1 per cent of the Fe^{59} IBP taken up by reticulocytes *in vitro* was in the supernatant solution of the hemolysate spun at 1,000 G. A small portion (4.5 per cent of the cell uptake) of the Fe^{59} in the original supernate was sedimented with the ribonucleic acid-rich microsome fraction, obtained by centrifuging the supernate at 105,000 G for 60 minutes. Another small portion of Fe^{59} (0.9 per cent of the cell uptake) was recovered in that fraction obtained by centrifuging the supernate at 26,360 G for 20

minutes and believed to contain the mitochondria. However, most of the nonmembrane Fe^{59} remained in the water-soluble phase of the lysed red cells and was incorporated into heme (see below). When a suspension of reticulocytes containing Fe^{59} derived from Fe^{59} IBP was washed free of Fe^{59} IBP and then incubated for up to 24 hours in native plasma or in 60 μM unsaturated IBP, there was no appreciable elution of Fe^{59} into the medium, and during this period the proportion of Fe^{59} in the supernate of the hemolyzed cells to that of the whole cells (62 per cent) remained unchanged.

Iron taken up from Fe^{59} IBP by a red cell suspension containing 32.2 per cent reticulocytes was not appreciably eluted by subsequent exposure to EDTA in pH 6.8 or pH 7.4 phosphate buffer, even at concentrations as high as 0.1 M EDTA (Figure 3). This was true both of intact Fe^{59}-labeled reticulocytes, in which approximately 55 per cent of the Fe^{59} was in the membrane (as determined by centrifugation of hemolysates), and of the Fe^{59} in cell membranes washed "free" of hemoglobin and then resuspended in EDTA. Thus the Fe^{59} was not unelutable simply because of incorporation into heme. On the other hand, all but 28 per cent of the iron taken up by the same red cell suspension (containing 32.2 per cent reticulocytes) from $Fe^{59}Cl_3$ in saline was eluted by EDTA. That

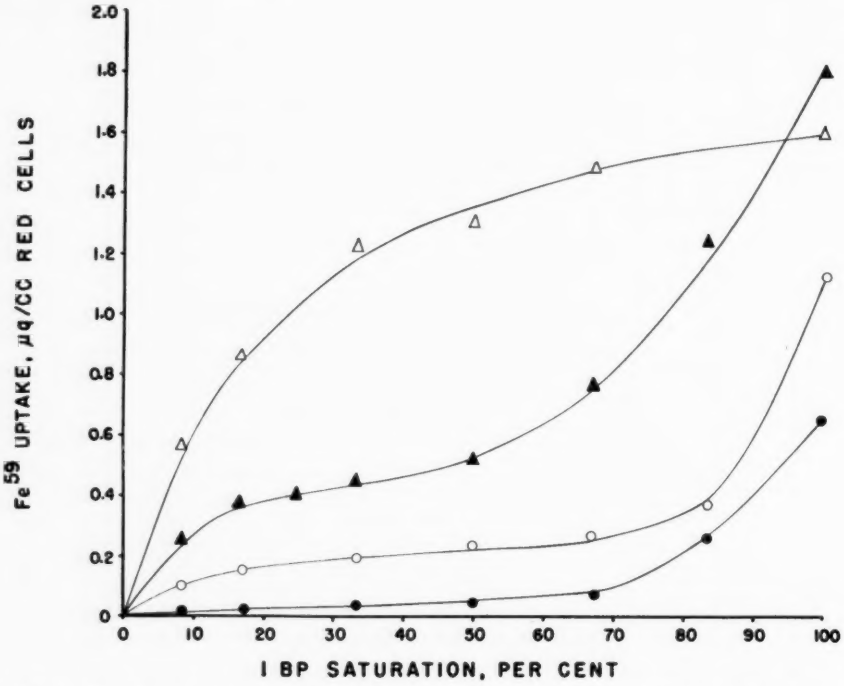

FIG. 4. THE EFFECT OF THE IRON SATURATION OF IBP UPON THE IRON UPTAKE OF RETICULOCYTE-CONTAINING RED CELL SUSPENSIONS FROM FOUR SUBJECTS

Note the tendency for Fe^{59} uptakes to level off at IBP saturations of over 20 to 30 per cent. If the iron uptake data above is plotted against the reciprocal of the IBP saturation (see Figure 5), straight-line relationships obtain at saturations from 0 to 50 per cent. The symbols represent red cells from subjects with the following hematologic conditions: acquired hemolytic anemia with 38.9 per cent reticulocytes (empty triangles); sickle cell anemia with 18.4 per cent reticulocytes (solid triangles); pernicious anemia during a therapeutic response with 14.9 per cent reticuloyctes (empty circles); and normal with 1.2 per cent reticulocytes (solid circles). Except with the acquired hemolytic anemia red cells a heavy "nonspecific" adsorption of iron on red cells became evident when the IBP saturation exceeded 60 to 85 per cent.

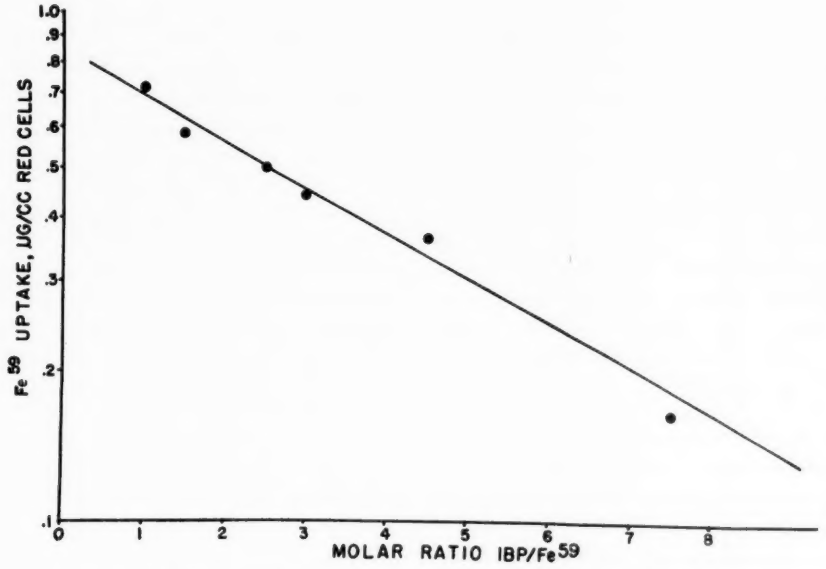

FIG. 5. THE COMPETITIVE EFFECT OF IBP ON IRON UPTAKE BY RETICULOCYTES

Each point represents the Fe^{59} uptake by equal volumes of reticulocytes incubated with constant amounts of Fe^{59} and increasing concentrations of IBP. Since each molecule of IBP is capable of binding two atoms of iron, the points represent IBP saturations ranging from 50 to 6 per cent. The expression "molar ratio IBP/Fe^{59}" is equivalent to half the reciprocal of the fractional iron saturation of IBP.

taken up by the mature red cells was almost entirely eluted by EDTA (Figure 3) as is true of other multivalent metals adsorbed onto red cells (30). Thus, the 28 per cent of the radioactivity that remained unelutable despite high concentrations of EDTA (Figure 3) was presumably bound to reticulocytes, but was not incorporated into heme, for 88 per cent of it was attached to the cell membranes.

In an effort to determine the chemical fraction of reticulocyte membranes to which Fe^{59} was bound, a reticulocyte-rich red cell suspension was incubated with autologus plasma, which had been brought to half-saturation with Fe^{59}. These cells were then washed five times with physiologic saline, after which they were hemolyzed in three volumes of cold water and later suspended in 50 volumes of cold acetate buffer for 18 hours. The stroma residue which precipitated from this suspension was subjected to lipid extraction by the method of Folch and associates (31). Less than 0.1 per cent of the Fe^{59} taken up by the cells was recovered in the lipid extract of their membranes, whereas 34.8 per cent remained bound to the stroma residue. This Fe^{59}-containing residue was partially soluble in 30 per cent sodium cholate. However, efforts to characterize the iron-binding component of this cholate-soluble extract by paper electrophoresis and by column chromatography were unsuccessful.

The transfer of iron from IBP to reticulocyte-rich suspensions of red cells was greatly affected by the degree of iron saturation of the IBP. In Figure 4 it is evident that the Fe^{59} uptake of reticulocytes approached a plateau when the iron saturation of the IBP exceeded 20 to 30 per cent. At iron saturations in excess of 60 to 85 per cent there was usually a sharp increase in Fe^{59} uptake; this appeared to be a nonspecific adsorption, for a similar sharp increase in Fe^{59} uptake also occurred when adult red cells were so studied. This "nonspecific" iron uptake at high iron saturation was partially diminished by increasing the bicarbonate concentration. Comparable experiments in which iron-deficient human plasma was saturated to various extents with iron revealed

that only when the plasma iron-binding capacity was exceeded did iron "nonspecifically" attach to red cells. Even when the iron-binding capacity of plasma was exceeded, however, a majority of the excess iron was bound by other proteins. As indicated by filter paper electrophoresis and autoradiography, iron added in excess of the plasma iron-binding capacity at an alkaline pH was largely bound to a protein migrating midway between the alpha-1 and alpha-2 globulins; greater excesses of iron also migrated with albumin. There was no difference in this respect between normal plasma and the plasma of patients with hemochromatosis.

When the IBP was less than about 20 per cent saturated, the iron uptake of reticulocytes diminished despite an amount of iron in the system well in excess of that utilizable by the reticulocytes. The competition of IBP for iron in the presence of a given volume of reticulocytes was demonstrated either by maintaining the IBP concentration constant and varying the amount of iron added (Figure 4) or by keeping the iron concentration constant and varying the amount of IBP (Figure 5).

When to a given amount of reticulocytes various quantities of IBP of a constant Fe^{59} saturation (33.3 per cent) were added, the Fe^{59} uptake increased in proportion to the IBP concentration until a concentration of 160 μM was exceeded (Figure 6). Thereafter, further increase in Fe^{59} IBP caused no further uptake.

In an effort to detect whether IBP entered reticulocytes or became attached to their surfaces, measurements were made of the IBP concentration of a preparation before and after exposure to a relatively large volume of reticulocytes. Despite removal by the reticulocytes of over 30 per cent of the iron, there was no change in the total IBP concentration.

The uptake by reticulocytes of Fe^{59} bound to various mammalian plasmas and to conalbumin

The uptake by human reticulocytes of iron from human plasma labeled with 1 μg. Fe^{59} per ml. plasma was similar to that from comparable concentrations and saturations of purified human IBP. As with human red cells, iron bound to 30 μM human IBP was not transferred to mature rabbit or rat red cells (iron uptake 0.03 μg. per

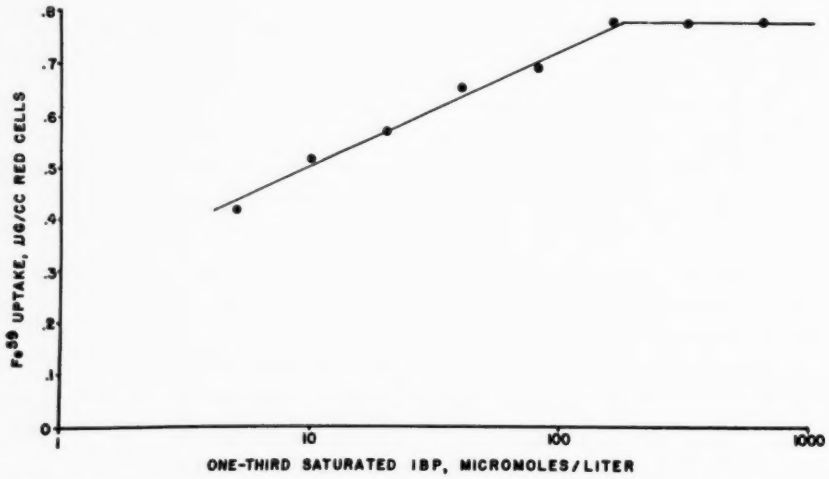

FIG. 6. THE EFFECT OF IBP CONCENTRATION ON THE UPTAKE OF IRON BY RETICULOCYTES

IBP which had been 33.3 per cent saturated with Fe^{59} was added in increasing concentrations to a series of test tubes holding equal volumes of a suspension of red cells containing 18.0 per cent reticulocytes. Iron uptake increased in direct proportion to the Fe^{59} IBP concentration until the latter exceeded 160 μM (1.39 Gm. per cent). The Fe^{59} uptake at physiologic concentrations of IBP (approximately 27 μM) was about 77 per cent of that at 160 μM concentrations of IBP.

TABLE I

Utilization of Fe^{59} by human reticulocytes

Iron preparation	Fe^{59} uptake	Fe^{59} incorporation into heme (2S)	Red cell Fe^{59} in heme	Fe^{59} incorporation into hemoglobin*	Red cell Fe^{59} in hemoglobin
	μg./ml. red cells	*μg./Gm. heme*	%	*μg./Gm. hemoglobin*	%
Fe^{59} IBP	0.24	15.6	76.5	0.485	64.6
$Fe^{59}Cl_3$	2.32	10.5	5.7	0.251	3.5

* Determined by autoradiography as described in the text.

ml. cells per two hours). A suspension of rabbit red cells containing 33.0 per cent reticulocytes, however, took up 0.60 μg. iron per ml. cells in two hours from human IBP, as compared to 1.4 μg. from rabbit plasma; and a suspension of rat cells containing 14.0 per cent reticulocytes took up 0.17 μg. iron per ml. red cells in two hours from human IBP, as compared to 0.37 μg. from rat plasma. Thus in each instance iron was transferred from human IBP to heterologous reticulocytes, albeit not as readily as from the native plasma.

Fe^{59} bound to 30 μM half-saturated conalbumin was not taken up by mature human red cells (0.006 μg. iron per ml. red cells in two hours). However, a preparation of human red cells containing 33.5 per cent reticulocytes took up 0.535 μg. iron per ml. red cells in two hours from 30 μM conalbumin, as compared to 0.613 μg. iron per ml. red cells in two hours from 30 μM human IBP. Thus, *in vitro*, conalbumin was approximately as efficient an iron transport vehicle for human reticulocytes as was human IBP. Conversely, chicken (nucleated) red cells (6.2 per cent reticulocytes) took up in two hours half as much iron (0.52 μg. per ml. red cells) from human IBP as they did from conalbumin (0.94 μg. per ml. red cells).

The fact that chicken red cells utilized iron from conalbumin somewhat more readily than from human IBP suggested the possibility that this protein, which normally is concentrated in hens' eggs, may also function as an avian plasma transport protein. As was intimated by Jensen, Ashenbrucker, Cartwright and Wintrobe (19), the addition of radioactive iron to fresh chicken plasma (which had an iron content of 160 μg. per cent) caused no colorimetric change. The added Fe^{59} was largely dialyzable thereafter and was "nonspecifically" adsorbable by both mature and immature human red cells. However, when native chicken plasma was dialyzed at pH 5.6 against EDTA to free it of iron, and then dialyzed free of EDTA, at a pH of 7.4, this iron-free plasma was found capable of binding about 200 μg. per cent of radioactive iron, as determined by dialysis experiments. Filter paper electrophoresis (0.05 M barbital buffer at pH 8.6) and autoradioautography of chicken plasma so labeled with Fe^{59} revealed a specific iron-binding protein with the same mobility as that of conalbumin: midway between the β and γ positions.

Incorporation of Fe^{59} into heme and into hemoglobin by human reticulocytes

In systems containing only labeled iron, IBP and washed reticulocytes suspended in saline and incubated at 37° C. for two hours, from 20 to 50 per cent of the Fe^{59} taken up by the reticulocytes was recoverable in the heme fraction obtained by the method of Fischer (29). Correcting for heme lost during fractionation, it was calculated in various experiments that from 30 to 80 per cent of the reticulocyte Fe^{59} was incorporated into heme. Despite the higher total cell uptake of Fe^{59} derived from $Fe^{59}Cl_3$ than from Fe^{59} IBP, the portion of this Fe^{59} which was recovered with heme was far less, ranging from 2 to 12 per cent and varying in direct proportion to the percentage of reticulocytes. Presumably this is due to the fact that mature red cells, which adsorb free iron but do not synthesize heme, constituted a majority of the cells in most suspensions studied. In a single experiment the incorporation of Fe^{59} from $Fe^{59}Cl_3$ and from Fe^{59} IBP into both heme, as determined by the Fischer method, and into hemoglobin, as determined by filter paper electrophoresis and autoradiography, was compared (Table I). Both methods were in essential agreement. Note that the calculated absolute amount of Fe^{59} incorporated from $Fe^{59}Cl_3$

into the heme fraction of reticulocytes was only about two-thirds of the amount of Fe^{59} incorporated into the heme of reticulocytes incubated with Fe^{59} IBP.

Factors affecting the transfer of iron from IBP to reticulocytes

a) Temperature. The effect of temperature on iron uptake by reticulocytes was determined by incubating reticulocyte-rich red cell suspensions and Fe^{59}-containing preparations together at certain temperatures, after these components had been separately equilibrated at these temperatures for 15 minutes. After incubation for two hours, the reaction was stopped by the addition of NaCN (see below), and the cells were then washed as usual. The uptake by reticulocytes of Fe^{59} bound to IBP was markedly temperature-dependent. As indicated in the upper part of Figure 7, there was very little uptake at 0.5° C.

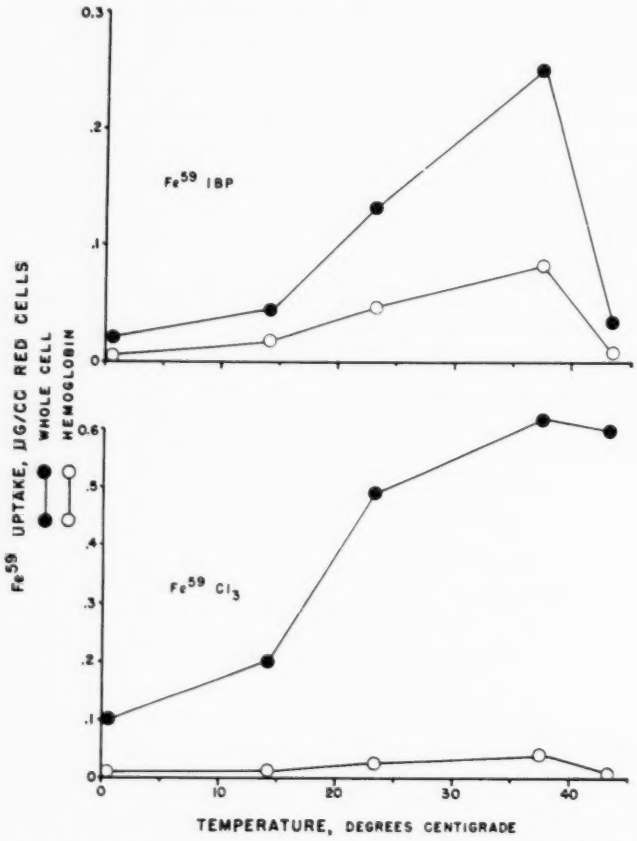

FIG. 7. THE EFFECT OF THE TEMPERATURE OF INCUBATION ON THE UPTAKE BY RETICULOCYTES OF Fe^{59} FROM Fe^{59} IBP AND FROM $Fe^{59}Cl_3$

In each instance the whole cell uptake of Fe^{59} (solid circles) increased with increases in temperature up to 37° C. However, Fe^{59} uptake from Fe^{59} IBP (above) was more strikingly suppressed at 0.5° C. and at 42.5° C. than that from $Fe^{59}Cl_3$ (below). In each study Fe^{59} incorporation into hemoglobin (empty circles), as determined by autoradiography, was also clearly depressed by nonphysiologic temperatures but an approximately constant proportion between hemoglobin Fe^{59} and whole cell Fe^{59} remained.

Above 14° C. iron uptake increased steadily to a maximum rate at 37° C.; at 42.5° C. the uptake was sharply inhibited. Based on these data the heat of activation (ΔE) was calculated as 12,200 calories per mole of iron. Almost identical temperature dependency was observed when Fe^{59} bound by plasma was incubated with reticulocytes. In parallel studies the uptake from $Fe^{59}Cl_3$ in saline both by reticulocyte-rich cell suspensions and by reticulocyte-poor cell suspensions also increased with rising temperatures, but this effect was less striking. The ratio of Fe^{59} uptake from Fe^{59} IBP at 37° C. over that at 0.5° C. was 12.6; the ratio of Fe^{59} uptake from $Fe^{59}Cl_3$ at 37° C. over that at 0.5° C. was 6.2. As seen in Figure 7 there was also a fall-off of the uptake of iron from $Fe^{59}Cl$ at 42.5° C., but this effect was slight compared to the sharp reduction when Fe^{59} IBP was employed. The incorporation of Fe^{59} from either $Fe^{59}Cl_3$ or Fe^{59} IBP into hemoglobin as determined by autoradiography showed a temperature dependence very similar to that of the whole cell uptake of Fe^{59} (Figure 7).

b) pH. The effect of pH on iron uptake was determined both in systems containing Fe^{59} IBP and in systems with Fe^{59}-labeled plasma. Both systems were adjusted to various pH levels with N/3 HCl or N/3 NaOH, and the final experimental pH level was determined after addition of the washed reticulocytes. A maximum of Fe^{59} uptake ranging from pH 7.2 to pH 7.6 was found in both systems, with sharp reductions outside of this range. Thus, there was very little iron uptake below pH 6.8 and above pH 8.0.

c) Oxygen tension and the state of oxidation. The effect of oxygen tension on iron uptake by reticulocytes *in vitro* was investigated, employing autologus plasma labeled with Fe^{59}. After removal by centrifugation of the red cells of blood samples of patients with pernicious anemia undergoing treatment with vitamin B_{12}, $Fe^{59}Cl_3$ was added to the native, hypoferremic, heparinized plasma in an amount calculated to half-saturate the iron-binding protein. Meanwhile, aliquots of the reticulocyte-rich red cells in unlabeled plasma were placed in a series of air-tight separatory funnels, each of which was equilibrated three times at one atmosphere pressure with one of the series of gas mixtures which contained various concentrations of O_2 in N_2 with a constant amount (4.7 ± 0.2 per cent) of CO_2. Fe^{59}-labeled plasma was then added to each separatory funnel and the mixtures were re-equilibrated three times with the same gas mixtures and incubated at 37°C. for three hours. As portrayed in the upper part of Table II, the iron uptake of the whole cells (presumably reticulocytes) was least under anaerobic conditions and increased somewhat with increasing O_2 tensions. On the other hand, the portion of red cell Fe^{59} which was present in the supernatant solution derived from hemolysates of these cells was greatest under anaerobic conditions and diminished with increasing O_2 tensions.

TABLE II

Effect of oxygen tension on the utilization of iron by human reticulocytes

pO_2	Subject A	B	C	D	Average
	Fe^{59} uptake (μg./ml. red cells)				
mm. Hg					
0	0.139	0.227	0.061	0.068	0.124
20				0.083	
39	0.176	0.234	0.075	0.082	0.142
81	0.224	0.243	0.082	0.090	0.160
113	0.212	0.252	0.079	0.089	0.158
144	0.210	0.261	0.084	0.091	0.162
724	0.210	0.273	0.086	0.095	0.166
	Per cent of red cell Fe^{59} in "hemoglobin"*				
0	42.3	81.2	47.6		57.0
20					
39	32.1	73.8	38.5		48.1
81	28.2	72.5	38.2		46.3
113	27.6	72.0	36.9		45.5
144	29.6	66.3	39.3		45.1
724	22.1	63.2	33.9		39.7

* Determined only presumptively in these experiments as that radioactivity remaining in the supernatant solution from centrifuged reticulocyte hemolysates.

Although it was possible to decolorize partially the iron-IBP complex with certain reducing agents such as sodium metabisulfite (2×10^{-2} M), cysteine (10^{-3} M) and glutathione (2×10^{-3}M), these agents did not facilitate Fe^{59} transfer from IBP to reticulocytes. Rather, Fe^{59} uptake by reticulocytes was somewhat diminished by chemical reduction, and was moderately increased by 5×10^{-2} M H_2O_2 and by 5×10^{-3} M $KMnO_4$.

d) Hemolysis. The effect of preliminary hemolysis of reticulocytes on their capacity to take up Fe^{59} from Fe^{59} IBP was determined by incubating Fe^{59} IBP with three aliquots of a reticulocyte-rich suspension of red cells, (a) in saline,

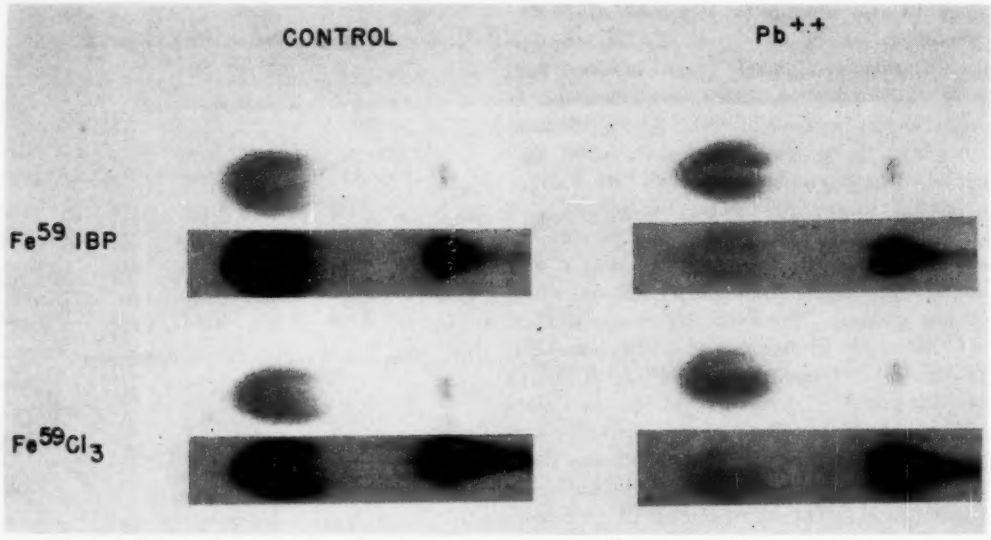

FIG. 8. THE EFFECT OF LEAD ON THE INCORPORATION BY RETICULOCYTES OF Fe^{59} FROM Fe^{59} IBP AND FROM $Fe^{59}Cl_3$ INTO HEMOGLOBIN

After incubation with Fe^{59}, human reticulocytes were washed free of unutilized iron prior to being hemolyzed and subjected to electrophoresis at pH 6.35 in 0.1 M phosphate buffer. The upper (lighter) of each of the four pairs of strips is the unstained hemoglobin paper electrophoretogram. In each the heavy spot to the left represents hemoglobin migrating toward the anode, and the small spot to the right is at the origin and consists largely of cell membranes. The lower (darker) of each of the four pairs of strips is the autoradiogram of the paper strip. As compared to incubation without lead (left), reticulocytes incubated with 10^{-4} M $PbCl_2$ (right) showed a marked inhibition of incorporation of Fe^{59} into hemoglobin, whether the Fe^{59} was derived from Fe^{59} IBP or $Fe^{59}Cl_3$. At this concentration of lead, however, the total reticulocyte uptake of Fe^{59} was only slightly inhibited, causing a relative accumulation of Fe^{59} on the reticulocyte membranes. Note in the control study how much more efficiently Fe^{59} was incorporated into hemoglobin when derived from Fe^{59} IBP than when derived from $Fe^{59}Cl_3$.

(b) after hemolysis with three volumes of distilled water, and (c) after hemolysis by rapid freezing and thawing. After incubation for two hours at 37° C. the intact reticulocytes in (a) were also hemolyzed with distilled water, and the cell membranes in all tubes were then washed three times with cold physiologic saline. The Fe^{59} content of the membranes from the reticulocytes hemolyzed in (b) and (c) before incubation with Fe^{59} IBP was only 7 per cent of that of membranes from reticulocytes in (a) which were intact during the incubation period.

c) Metabolic accelerators and inhibitors. The influence of several metabolic accelerators and substrates on the transfer of Fe^{59} from buffered solutions of Fe^{59} IBP to human reticulocytes was evaluated during two hour periods of incubation at 37.5° C. Glucose in concentrations of 2×10^{-2} M increased Fe^{59} uptake by reticulocytes moderately, with values ranging from 110 per cent to 150 per cent of control values. Inosine (5×10^{-2} M), glycine (10^{-1} M), succinic acid (10^{-1} M), methylene blue (20 mg. per cent) and citrate (10^{-1} M), all in the presence of 2×10^{-2} M glucose, did not increase Fe^{59} uptake further, either singly or in combination.

The uptake of Fe^{59} from Fe^{59} IBP by reticulocytes was moderately depressed, but not abolished by HCO_3^- concentrations in excess of 10^{-2} M. At given HCO_3^- levels, sodium acetazoleamide at concentrations as high as 50 mg. per ml. cells had no effect on iron uptake. Each of several "metabolic inhibitors" studied (10^{-2} M sodium cyanide, 10^{-2} M sodium fluoride, 10^{-2} M sodium azide, 10^{-3} M sodium arsenate and 10^{-3} M iodoacetic acid) reduced the Fe^{59} uptake by reticulocytes (suspended in Krebs-Ringer phosphate so-

lution containing glucose) to less than 20 per cent of control values. Marked inhibition of iron uptake was caused by as little as 10^{-4} M concentrations of 2,4-dinitrophenol and 10^{-5} M 2,4-dinitrophenylphenol. However, concentrations of fluoracetate as high as 2×10^{-2} M had no inhibitory effect.

The possibility that iron uptake was suppressed indirectly by inhibition of heme synthesis was examined by observing the effect of lead, which interferes with several stages in the synthesis of heme (32, 33). Although inhibited by 5×10^{-4} concentrations of Pb^{++}, the whole cell uptake of Fe^{59} by human reticulocytes during two hours at 37.5° C. was only slightly diminished (− 20.6 per cent) in the presence of 1×10^{-4} M Pb^{++}. However, the incorporation of Fe^{59} into hemoglobin was almost entirely prevented, as is evident in Figure 8, whether the Fe^{59} was derived from Fe^{59} IBP or from $Fe^{59}Cl_3$ in saline. Thus the amount of iron remaining in the cell membrane was increased. This pile-up of iron on the membrane was not the result of an increase in membrane hemoglobin and the increased membrane Fe^{59} in lead-poisoned reticulocytes was not recovered in heme extracts of the membrane.

The results of a comparable study of the effect of lead on the uptake of Fe^{59} from rabbit plasma by the reticulocytes of bled rabbits are presented in Table III. In this experiment a reticulocyte-rich suspension of washed rabbit red cells was incubated for five minutes with 10^{-4} M $PbCl_2$, after which Fe^{59}-labeled plasma was added and incubated with the cells at 37°C. under oxygen for one hour before cell fractionation was carried out. It is evident from Table III that, although the total cell uptake of Fe^{59} was moderately diminished by this concentration of Pb^{++}, there was a striking increase of Fe^{59} in the stromal (membrane) fraction of lead-poisoned cells, and, proportionately, a reduction in Fe^{59} in the hemoglobin-containing supernate. There was no evidence that lead caused iron to accumulate in the microsomal fraction or in the fraction believed to contain the mitochondria.

f) Metals and chelating agents. The question whether, in its transfer from IBP to reticulocytes, iron exists at any time in the free ionized state was examined by adding Fe^{59} IBP to each of a series of tubes containing a reticulocyte-rich suspension of red cells to which various amounts of nonradioactive $FeCl_3$ had been added. There was no competitive inhibition of Fe^{59} uptake by the added ionized iron. The effect on iron uptake of several other metals known to participate in enzymic systems was also explored. Concentrations of KCl, $MgCl_2$, and $CaCl_2$ ranging from 10^{-4} to 10^{-2} M had no effect. $CoCl_2$ concentrations of 10^{-3} M, or greater, moderately suppressed iron uptake.

More evidence that iron did not exist in the free state during transfer from IBP to reticulocytes, and that traces of nonferric metals were not involved in the reaction, was obtained by use of EDTA which competes with IBP for iron. As is shown in Figure 9, EDTA dissolved in pH 7.4 phosphate buffer completely blocked the uptake by red cells of Fe^{59} from $Fe^{59}Cl_3$ at a molar ratio of EDTA/Fe of two. EDTA was equally effective in this respect on reticulocytes (Figure 9) and on adult red cells (not shown), indicating that it did not function as an iron transfer agent. In contrast to the findings with $Fe^{59}Cl_3$, the transfer of iron from IBP to reticulocytes was not significantly affected by the presence of EDTA until the ratio of EDTA/Fe exceeded 10; and Fe^{59} uptake from Fe^{59} IBP was not abolished even at a ratio of EDTA/Fe of 100, despite the fact that

TABLE III

The effect of lead on the uptake and intracellular distribution of iron in rabbit reticulocytes

		Fe^{59} activity (*cpm/ml. cells*)			
		Sediment from spun hemolysate			
Reticulocyte suspension	Whole cells	7,000 G × 15 min. (Cell membrane)	26,000 G × 20 min. (Mitochondria)	105,000 G × 60 min. (Microsomes)	Soluble supernate
Control	446,250	23,200	800	700	412,200
Lead-poisoned (10^{-4} M Pb^{++})	210,200	113,100	1,600	900	76,720

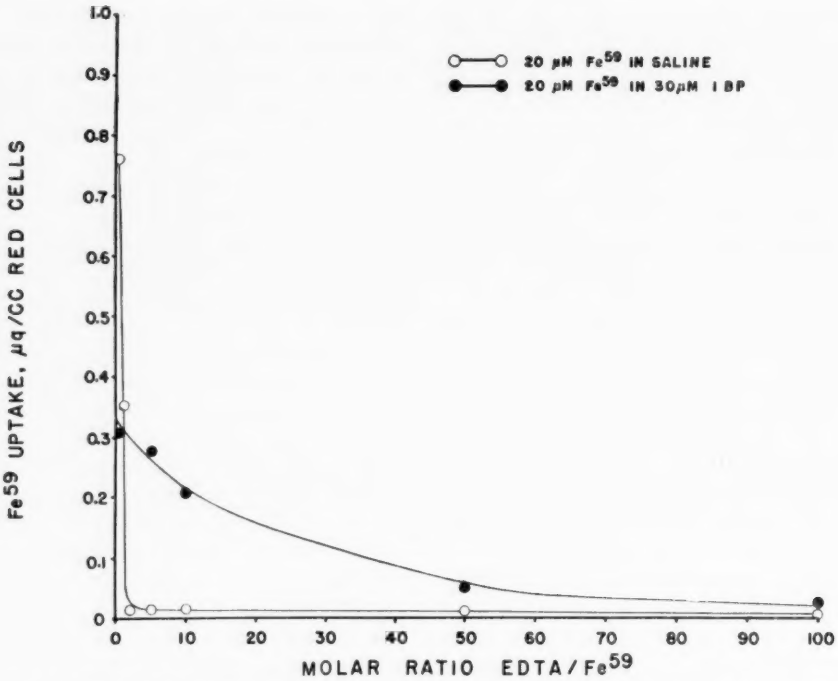

FIG. 9. THE EFFECT OF EDTA ON THE UPTAKE OF Fe^{59} BY RETICULOCYTES FROM Fe^{59} IBP AND $Fe^{59}Cl_3$

With a molar ratio of EDTA/iron of two or more, iron was not taken up from $Fe^{59}Cl_3$ by reticulocytes. However, a relatively large excess of EDTA was needed to suppress the transfer of iron from Fe^{59} IBP to reticulocytes.

this amount of EDTA caused over 50 per cent dissociation of the Fe^{59} IBP complex as determined colorimetrically.

g) Effect of enzymatic alteration of red cell surfaces. In order to explore the hypothesis that the immature red cell membrane includes on its surface receptor sites which bind iron, the effects of certain enyzmes which alter the red cell surface were observed. Reticulocyte-rich washed red cell suspensions were incubated for one hour at 37° C. in pH 7.6 phosphate buffer with various concentrations of trypsin.[7] These cells were then washed in phosphate buffer (pH 7.4) once, and in physiologic saline twice, and were then incubated, in parallel studies, with Fe^{59} IBP and with $Fe^{59}Cl_3$ in saline. Pretreatment of reticulocytes with as little as 0.001 per cent trypsin (0.01 mg. per ml.) markedly suppressed their ability to take up Fe^{59} IBP (left portion of Figure 10). On the other hand, the adsorption of $Fe^{59}Cl_3$ by these enzyme-treated cells was unaffected. As indicated by the interrupted line in Figure 10, the uptake of iron from Fe^{59} IBP by trypsinized reticulocytes was consistently greater (although still markedly depressed) when glucose (0.015 M) was included in the system during incubation of cells and Fe^{59} IBP. When reticulocytes were first trypsinized, then incubated with glucose for two hours, and finally incubated with Fe^{59} IBP, with two saline washings of the cells between each incubation, the effect of trypsinization upon iron uptake was again diminished but not to the extent observed when glucose was present during the incubation of cells with Fe^{59} IBP. The presence of iron in the medium during trypsinization did not affect the subse-

[7] Trypsin, crystalline (salt-free, lyophilized). Mann Research Laboratories, New York, N. Y.

quent inhibition of Fe^{59} uptake. Trypsinization of reticulocytes curtailed the subsequent uptake of Fe^{59} from Fe^{59} bound to plasma to the same extent as that bound to purified IBP. Reticulocytes, previously incubated with various concentrations of trypsin as described above, were resuspended in plasma containing Fe^{59} and were then incubated in Warburg flasks for three hours. During this time oxygen consumption was observed, and at the end of incubation the lactate content of the supernatant plasma specimens and the Fe^{59} activity of the red cell specimens were determined. Although Fe^{59} uptake by the reticulocytes was, once again, sharply diminished by pretreatment with 0.001 per cent. 0.01 per cent, and 0.1 per cent trypsin, these concentrations of trypsin had no effect upon either oxygen consumption or lactate production. Incubation of reticulocytes with 0.1 per cent trypsin, after these cells had taken up Fe^{59} from Fe^{59} IBP, did not cause elution of Fe^{59} into the medium, even though 50 per cent of the Fe^{59} was in the cell membranes as determined by centrifugation. Chymotrypsin [8] treatment also blocked the uptake of Fe^{59} by reticulocytes, although it was not quite as potent in this respect as trypsin.

The effect of enzymatic destruction of the sialic acid of the reticulocyte surfaces by receptor-

[8] Chymotrypsin, crystalline. Mann Research Laboratories, New York, N. Y.

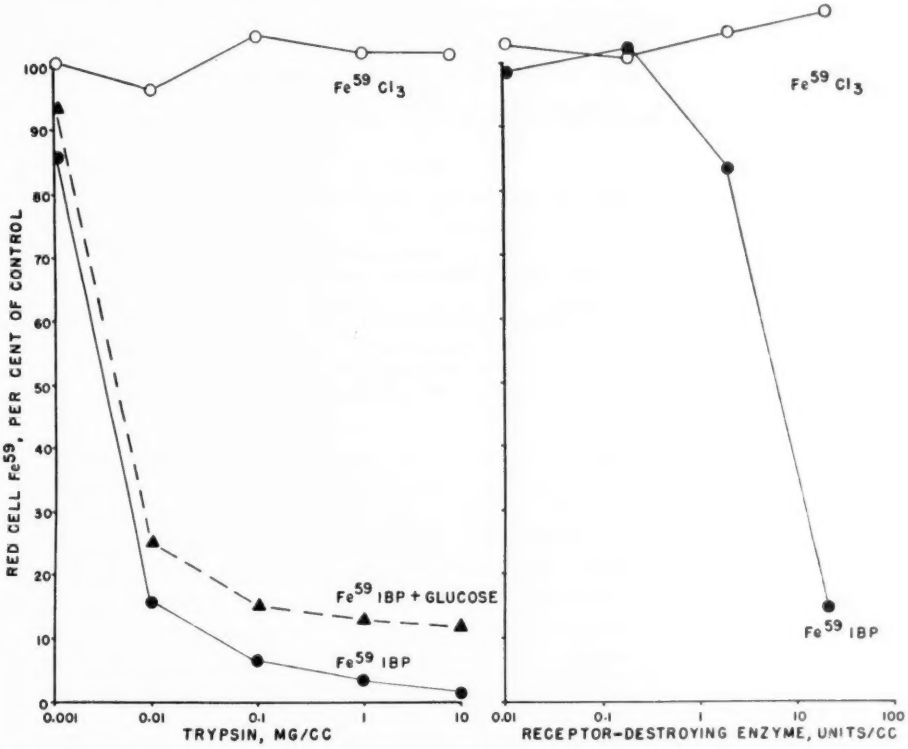

FIG. 10. THE EFFECT OF ENZYMATIC ALTERATION OF RETICULOCYTE SURFACES ON THEIR SUBSEQUENT ABILITY TO TAKE UP IRON FROM $Fe^{59}Cl_3$ AND Fe^{59} IBP

Neither trypsin (left) nor receptor-destroying enzyme (RDE) (right) significantly affected the uptake by reticulocytes of Fe^{59} from $Fe^{59}Cl_3$. Exposure of reticulocytes to extremely low concentrations of trypsin, however, caused a marked suppression of their uptake of iron from either purified IBP (left) or from plasma (not shown). This effect of trypsin was partly reversed by the presence of glucose (interrupted line). RDE also diminished the subsequent uptake of reticulocytes of Fe^{59} from Fe^{59} IBP. However, the quantity of RDE preparation required to cause inhibition far exceeded that of trypsin.

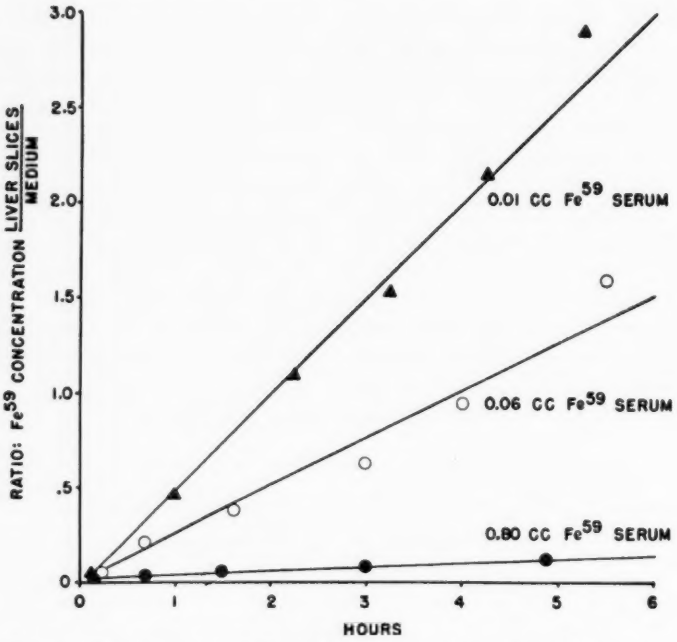

FIG. 11. THE UPTAKE OF Fe^{59} FROM RAT SERUM BY RAT LIVER SLICES AS A FUNCTION OF TIME

Rat liver slices took up iron from rat serum in direct proportion to the period of incubation. Only when comparatively small amounts of Fe^{59}-labeled rat serum were present, however, was an actual concentration gradient achieved. Thus when five rat liver slices were incubated with 0.01 ml. of Fe^{59} serum for six hours (solid triangles), the concentration of Fe^{59} in the slices was 3.1 times that of the medium. However, when 0.8 ml. of Fe^{59} serum was present (solid circles) the concentration of liver slice Fe^{59} was only 0.15 that of the medium.

destroying enzyme (34) was investigated. Lyophilized "Receptor-Destroying Enzyme" (RDE),[9] derived from vibrio cholera filtrates by the method of Ada and French (35), and quantitated in the manner of Burnet, McCrea and Stone (36), was dissolved in saline, and various amounts were added to a series of tubes containing buffered saline (pH 6.6) and 0.002 M $CaCl_2$. Washed suspensions of reticulocyte-rich red cells were incubated with the RDE preparations, and the cellular uptake of Fe^{59} was then determined as described above for the experiments with trypsin. Like trypsin, RDE had no effect upon the ability of red cells to adsorb Fe^{+++} from $Fe^{59}Cl_3$ in saline. However, at concentrations of this enzyme exceeding 1 unit per ml., reticulocytes were impaired in their ability to take up iron from Fe^{59} IBP (Figure 10). Nevertheless, compared to trypsin, much greater concentrations of the RDE preparation were required to inhibit such iron uptake. Thus, 240 mg. of RDE protein per ml. red cells was required to suppress iron uptake to the degree effected by 0.01 mg. of trypsin per ml. red cells. Preincubation, in an analogous manner, of reticulocyte-rich red cells and potassium periodate (KIO_4), which also inactivates sialic acid and related substances (37), caused a moderate (27 per cent) diminution of subsequent iron uptake by the reticulocytes from Fe^{59} IBP. However, the concentration of KIO_4 (10^{-3} M) required to accomplish this inhibition was very close to that concentration (2×10^{-3} M) causing hemolysis.

[9] Behringwerke, Marburg-Lahn, Federated Republic of Germany.

Comparisons between the uptake of Fe^{59} by rat reticulocytes and rat liver slices

The mechanism of uptake of iron from IBP by a nonerythroid, iron-requiring tissue was explored by utilizing rat liver slices. Livers, freshly obtained from decapitated Lobund rats, were cut with a Stadie-Riggs microtome into slices weighing 50 to 100 mg. The weighed slices were then placed in groups of five in iron-free flasks containing 2 ml. of Krebs-Ringer-phosphate solution to which glucose had been added. After equilibration by agitation at 37° C. under 95 per cent oxygen and 5 per cent CO_2, 1 ml. volumes of autologous rat serum which had been half-saturated with Fe^{59} and diluted with saline were added to each flask. Appropriately, at various time intervals, the supernate of each selected flask was decanted and saved, and its liver slices were rinsed 10 times in 50 volumes of saline. The Fe^{59} activities of each supernate and of the corresponding slices were then determined and expressed as the ratio of the Fe^{59} concentration per gram of liver slice to that per milliliter of medium.

As shown in Figure 11, rat liver slices accumulated Fe^{59} at a steady rate during six hours' incubation. However, in this system a concentration of Fe^{59} by liver slices exceeding that of the medium was only manifest after several hours when the Fe^{59} serum was present in relatively small amounts (< 0.1 ml. serum, half-saturated with Fe^{59}, per flask). Fe^{59} accumulated by rat liver slices did not leak out if the slices were subsequently incubated with rat serum half-saturated with nonradioactive iron. On the other hand, high concentrations of human IBP (50 μM) removed two-thirds of the Fe^{59} from rat liver slices within three hours, although this concentration of IBP failed to remove Fe^{59} from the stroma of rat reticulocytes. Preliminary boiling of rat

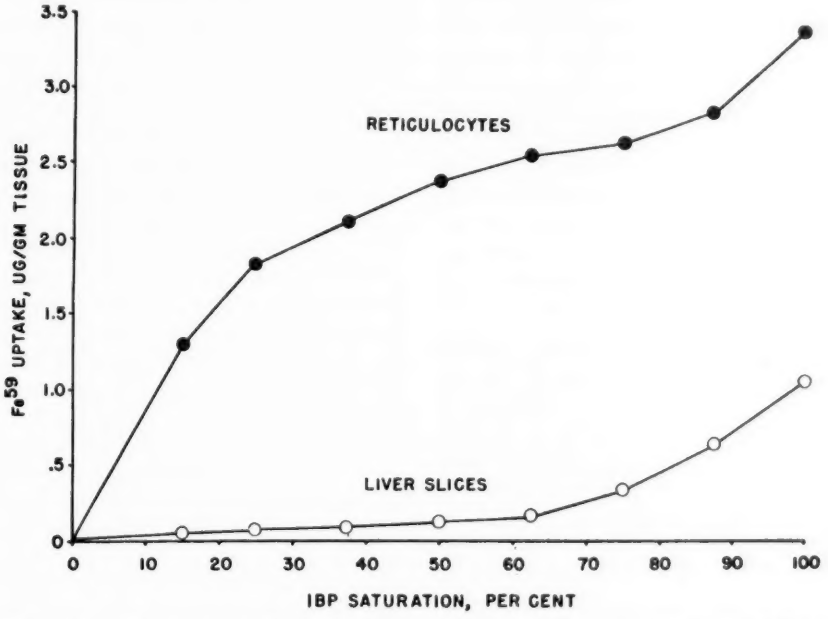

FIG. 12. THE EFFECT OF THE IRON SATURATION OF HUMAN IBP ON THE UPTAKE OF Fe^{59} BY RAT RETICULOCYTES AND BY RAT LIVER SLICES

As with human reticulocytes, the uptake of Fe^{59} from IBP by reticulocytes approached a plateau at an iron-binding protein saturation of 20 or 30 per cent, and a further sharp increase occurred at saturations above 75 per cent. In contrast rat liver slices took up comparatively small amounts of Fe^{59} until high saturations (above 62 per cent) were reached, when Fe^{59} adsorption sharply increased.

liver slices for five minutes not only failed to diminish the subsequent accumulation of Fe^{59} from plasma by liver slices, but actually augmented it moderately. Boiling of rat reticulocytes, on the other hand, virtually abolished their capacity to take up iron.

A comparison between the uptake of iron by reticulocytes and by liver at various iron saturations of IBP was made by separately incubating autologous rat reticulocytes and liver slices, respectively, with purified human IBP which had been incubated with various amounts of $Fe^{59}Cl_3$. The reticulocytes and liver slices were taken from a rat which had been bled 20 ml. per Kg. body weight three and four days before sacrifice. As depicted in Figure 12, rat reticulocytes resembled human reticulocytes in that the Fe^{59} uptake tended to level off when the iron saturation of the IBP preparation exceeded 20 to 30 per cent, only to rise further as full saturation was approached. With liver slices, on the other hand, Fe^{59} uptake increased slightly in a linear relationship with IBP saturation until, again, heavy deposition of Fe^{59} appeared at high saturations. It is apparent from this figure that at low IBP saturations the relative capacity of reticulocytes to take up iron was great, as compared to liver slices, whereas at high saturations of IBP the relative ability of liver slices to take up iron increased sharply.

The uptake of cobalt by mature and immature red cells

Cobalt, like most multivalent metals, combines with the membranes of normal washed red cells (30). Unlike iron or chromium, however, cobalt (as saline solutions of $Co^{60}Cl_2$ or $Co^{60}Cl_3$) was invariably taken up more avidly by reticulocytes than by adult cells. The red cells of several patients with increased reticulocytes were centrifuged and samples were removed from the top fractions (which contain most of the reticulocytes), the middle fractions, and the bottom fractions (said to contain the oldest cells [38]). In Table IV, it is apparent that the most immature red cells took up Co^{++} most extensively, and that Co^{++} uptake was roughly inversely proportional to cell age. Cobalt oxidized to Co^{+++} by the presence of 10^{-2} M H_2O_2 was similarly accumulated preferentially from a saline solution by immature red cells. In suspensions of reticulocytes and of adult red cells, the uptake of Co^{+++} was 1.47 and 1.38 times, respectively, that of Co^{++}, following the rule that the more highly charged cations are more extensively bound by red cell membranes (30).

TABLE IV

The uptake of Co^{++} by mature and immature red cells

Type of red cells	Centrifuged fraction	Reticulocytes	Conc. $Co^{60}Cl_2$	Cobalt uptake
		%	μM	*μg./100 ml. red cells*
Pernicious anemia	Top	59.1	30	20.48
	Middle	1.6		10.87
	Bottom	0.8		5.36
Pernicious anemia	Top	8.6	10	8.74
	Middle	3.4		6.55
	Bottom	0.4		4.77
Pernicious anemia	Top	30.2	10	5.70
	Middle	12.6		5.31
	Bottom	0.5		3.87
Acquired hemolytic anemia	Top	39.9	10	13.17
	Middle	26.1		8.73
	Bottom	17.5		4.60

Co^{+++} reacts with IBP to form an amber-colored compound with a light absorptive maximum at 402 mμ (6). This compound is stable as long as the cobalt is maintained in the trivalent state by a suitable oxidant, and unlike Cu^{++} and Zn^{++}, Co^{+++} is not displaced from IBP by Fe^{+++}. When Co^{60} IBP was incubated with adult red cells, little Co^{60} became attached to the red cell membrane until the IBP was more than half-saturated. When the cobalt saturation of IBP was less than 50 per cent, however, the Co^{60} uptake of reticulocyte-rich red cell suspensions was from 5- to 12-fold as great as that of adult red cells. It is to be noted that this was an even greater degree of preferential binding by reticulocytes than occurred with ionized cobalt. However, the uptake of Co^{60} from Co^{60} IBP by reticulocytes was considerably less than that of Fe^{59} from comparable preparations of Fe^{59} IBP, averaging about one-tenth as much.

Colorimetric interference by serum made it difficult to determine by spectrophotometry whether or not Co^{+++} in the presence of H_2O_2 formed a colored complex with the IBP or iron-deficient serum. However, with several specimens of human and of rat sera it was possible

to show that $CoCl_3$, added serially to plasma in a manner comparable to that employed in the determination of the serum iron-binding capacity (27), produced small increments in light absorption at a wavelength of 400 mμ. The erythropoietic effect of Co IBP *in vivo* was studied in four rats. Each was injected intravenously twice weekly for eight weeks with 2.5 ml. volumes of rat plasma which had been freshly incubated for one hour in the presence of H_2O_2 and sufficient $CoCl_2$ (10 μg.) theoretically to saturate fully the plasma IBP. No hematologic change occurred in rats injected with small amounts of cobalt in this form.

DISCUSSION

In common with most multivalent metals (30), iron in the free cationic form is adsorbed onto the membranes of either mature or immature red cells. This adsorption is injurious to red cells and may lead to their agglutination and even to hemolysis (30). Although both mature and immature red cells may take up free Fe^{+++}, only the immature cells have the capacity to incorporate this membrane-bound iron into heme. The nonspecific capacity of red cell membranes to absorb cations such as iron presumably explains an observation by Ellis, Brandt and Thacker (39) that more iron was taken up *in vitro* by washed than by unwashed reticulocytes with which serum binding of iron would be in competition.

The mature red cells from patients with acquired hemolytic anemia whose red cells are agglutinable by Coombs serum, as well as red cells sensitized with incomplete anti-D antibodies, take up less free Fe^{+++} than do normal red cells, suggesting that those chemical groups on the red cell membrane reacting with Fe^{+++} are partially blocked by such protein "coatings."

In contrast to free cationic iron, iron bound to human serum IBP was readily utilized by immature red cells but was completely unavailable to mature red cells. In this sense the iron transporting protein of plasma is involved in a remarkable mechanism for restraining iron from reacting with, and perhaps causing injury to, cells not requiring iron while making iron available to cells which utilize iron. In comparing several substances with IBP in terms of the selectivity with which iron bound to them was utilized by reticulocytes and not by mature red cells, albumin was found to be much less selective, and gamma globulin and EDTA did not function in selective transport capacity at all.

The transfer of iron from IBP to reticulocytes is not species specific. Indeed, it is not even class specific, since human reticulocytes readily utilized iron bound to conalbumin, the iron-binding protein of egg white, although conalbumin does inhibit iron utilization by bacteria (9). Conversely, a recent report by Paoletti (40) indicates that avian red cells are able to utilize iron from a preparation of human IBP. These observations indicate that the structure of the protein bearing the iron-chelating groups of IBP is not critical to the transfer of iron to immature red cells. On the other hand, the iron-chelating rings of IBP and of conalbumin are believed to be very similar (12).

The transfer of iron from IBP to immature red cells did not approach a maximum until the iron saturation of the protein reached 20 or 25 per cent, and nonspecific "unloading" of iron from IBP on to mature red cells began to occur at saturations above 60 per cent. It is therefore of interest that these are approximately the lower and upper limits, respectively, of normal IBP saturation in man. Thus, it is tempting to conclude that these homeostatic limits are preserved because below a saturation of 20 per cent IBP competes successfully with the marrow for iron, while above 60 per cent saturation iron is unloaded onto tissues having comparatively less affinity for iron. Experimental support for such a mechanism in the regulation of serum iron levels is provided in the comparative study of iron uptake by rat reticulocytes and rat liver slices. It is apparent in Figure 12 that at high iron saturations of IBP there is a sharp increase in the proportion of iron deposited in liver as compared to reticulocytes. The inability of liver slices, as compared to reticulocytes, to take up iron at low IBP saturations may explain the observation of Beutler (41) that rat liver cytochrome C levels are more strikingly reduced during mild or modest iron deficiency than are hemoglobin levels.

Since the total IBP concentration of serum increases during iron deficiency as well as during pregnancy (42), with consequent lowering of the percentage iron saturation, the resultant more

active competition by the more unsaturated IBP for iron (Figure 5) might be considered disadvantageous to the immature red cells. A similar situation pertains to serum thyroxine-binding protein, the levels of which increase in myxedema and in pregnancy (43). Thus, this transport protein also appears to compete with tissues for the transported metabolite (44). It is, of course, possible that the levels of these two proteins are homeostatically adjusted primarily with respect to achieving maximum loading rather than unloading of iron and thyroxine, respectively.

The reticulocyte membrane acts in a role intermediate between the stage of plasma transport and heme synthesis, as suggested by Walsh and his associates (15), and may be considered the site of an iron-trapping mechanism. It is clear that IBP, with a biological half-life of at least 12 days (45), is not consumed during the cellular utilization of iron, which normally is half-cleared from the plasma in less than two hours. Although theoretically iron may be released from IBP by reduction, a drop in pH, or removal of HCO_3^-, it is also clear from the studies with EDTA reported here that, in transfer, iron is not released from IBP for any appreciable time or distance as a free ion but is directly and intimately transferred from IBP to the reticulocyte membrane. No evidence was obtained that IBP itself entered or became attached to the surfaces of the reticulocytes, although a momentary physical interaction of this sort would be hard to exclude. It is probably more reasonable to assume that IBP molecules in close proximity to the reticulocyte membrane relinquish iron to specific iron-binding receptors on the cell surface. Consistent with this explanation is the finding of a competition between IBP and reticulocytes for iron, *in vitro,* and the striking inability of reticulocytes to take up iron from IBP after these cells had been exposed to very small concentrations of trypsin and chymotrypsin. It is not possible to assign any specificity to this action of trypsin except to note that extremely low concentrations of trypsin were effective, and that the respiratory and glycolytic activities of the reticulocytes were unaffected by these concentrations. Although trypsin readily lyses the neuraminic acid group of substances, such as occur prominently on red cell surfaces (34, 46), the comparatively weak effect of "receptor-destroying enzyme" and of periodate in inhibiting iron uptake by reticulocytes indicates that the iron receptors of red cells are not identical with those red cell receptors which react with many viruses and which consist primarily of *n*-acetylneuraminic (sialic) acid (34, 47, 48). The inability to remove more than 5 or 6 per cent of the Fe^{59} from reticulocyte membranes with high concentrations of IBP or EDTA is presumably attributable to the rapid incorporation of membrane iron into undissociable complexes.

It is difficult to say whether immature red cells differ from mature red cells in their ability to trap iron because immature red cells possess unique receptors or because they alone are able to metabolize membrane iron further, leaving such receptors continually unsaturated. Tishkoff, Robscheit-Robbins and Whipple have shown that the red cell stroma of dogs contains 12.4 μg. of iron per ml. red cells and that most of this is hemin iron (49). Human mature red cell stroma was found by Tishkoff normally to contain less than 1 μg. of nonhemin iron per ml. red cells, although in certain patients with erythroblastemia stromal iron levels were distinctly higher (50). The concentration of iron in the stroma of immature red cells at various stages of development is not known, but it is known by optical microscopy and, recently, by electron microscopy (21, 22) that the stroma of immature red cells, including reticulocytes, normally contains numerous iron-rich granules. Many of these granules have the aspect of ferritin (1) by electron microscopy (21, 22). It thus seems reasonable to suppose that in its stromal phase iron may exist in part in the form of ferritin, although, using chemical methods, we have been unable to demonstrate this. Nevertheless, it is probable that the proportion of iron localizing in the reticulocyte membranes in the experiments reported here far exceeds that which occurs *in vivo.* Whereas the mechanism for trapping iron remains relatively intact for several hours *in vitro,* it is presumed that the mechanisms for metabolizing the iron further partially break down.

Certain bacteria and smut fungi which require iron for the synthesis of cytochrome C and other iron-containing substances produce organic compounds with a strong affinity for iron when

grown in an iron-deficient medium[10] (51). It is believed that these organic compounds serve as carriers for the transfer of ferric ions from the medium into the bacterial cell for use in heme synthesis (51) and are analogous to the mineral-dissolving organic compounds excreted by certain higher plants capable of growing on rocks (52). Neilands (51) found several compounds which, when added to simple media, served to render iron available for bacterial utilization; these included citric acid and fructose-phenylalanine. The function of the latter in this respect is of interest, since red cell membranes contain ketose-amino acids (46) and since Borsook and his associates (53, 54) have reported that ketose-amino acids stimulate the incorporation of amino acids into the proteins of reticulocytes when added in the presence of iron. In order to examine the possibility that ketose-amino acids participate in the transfer of iron from IBP to immature human red cells, fructose-phenylalanine, fructose-glycine and fructose-glutamine were prepared by the method of Abrams, Lowy and Borsook (55). The effect of various concentrations of these substances on Fe^{59} uptake from Fe^{59} IBP by suspensions of mature red cells, of immature red cells and of trypsinized immature red cells was then assessed. In no instance was there an acceleration of iron uptake or of iron incorporation into hemoglobin by these cells. These negative findings fail to support, but do not exclude, the possibility that such compounds, in proper steric arrangement, may function as iron-binding or iron-carrying components of the membranes of immature red cells. It is reasonable to suppose that the iron-binding substances manufactured by iron-deficient bacteria and fungi are designed merely to render inorganic iron in the surrounding medium soluble and diffusible, and that the evolution in animals of cell receptors capable of directly removing iron from IBP supplanted that mechanism. Bacteria, on the other hand, ordinarily have no need for such receptors, and indeed do not possess them, as indicated by their inability to utilize conalbumin-bound iron (9).

Since the uptake of Fe^{59} from Fe^{59} IBP was only moderately diminished by anoxia *in vitro*, it is improbable that iron transfer is directly dependent upon oxidative mechanisms. However, the effect of temperature upon iron uptake by reticulocytes, with striking inhibition of uptake at temperatures only a few degrees above or below 37° C., is suggestive of an active metabolic mechanism.[11] This conclusion is supported by the finding that iron uptake was stimulated by the presence of glucose and was readily inhibited by a number of agents which block intermediary carbohydrate metabolism, including cyanide, azide, fluoride, arsenate, 2,4-dinitrophenol and 2,4-dinitrophenylphenol. Immature red cells are well known to be metabolically active, and reticulocytes have been shown to possess numerous enzymes, including various elements of the tricarboxylic acid cycle (57), and these are situated at least in part in the cell membrane (57, 58). Since hemolysis was found here to block the transfer of Fe^{59} from Fe^{59} IBP to reticulocytes, it is of interest that Hofmann and Rapoport (59) found that the respiration of reticulocytes *in vitro* was considerably reduced by hemolysis, probably by disruption of the tricarboxylic acid cycle. One may reasonably conclude that the uptake of iron by immature red cells is dependent at some stage upon metabolic energy, although this energy may not be directly required for the actual transfer of iron from IBP. In its dependence upon metabolic energy, iron uptake from IBP by immature red cells differs from that by rat liver slices. Saltman, Fiskin, Bellinger and Alex (60) have reported that rat liver slices accumulate iron against a concentration gradient, and that this accumulation is unaffected by metabolic inhibitors. However, the iron employed in those studies was not bound to IBP, and the findings are more analogous to those described here with red cells in which unbound iron was used.

The fact that appropriate concentrations of

[10] Interestingly, the smut fungus, U. sphaerogena, becomes pale and releases iron-binding compounds into the medium when the iron concentration of the medium falls to levels similar to those in the sera of iron-deficient patients (25 μg. per cent); these compounds disappear when the iron concentration is raised to that of normal sera (> 100 μg. per cent) (51).

[11] Although calculation of the heat of activation of a complex and incompletely-understood reaction as is involved here is very crude, the value for heat of activation calculated from the data in Figure 6 is within the range encountered in "active metabolic" or "enzymatic" reactions (56).

lead block iron incorporation into the hemoglobin of reticulocytes without blocking the cellular uptake of iron from IBP indicates that the rate of transfer of iron from IBP is not directly dependent upon the rate of heme synthesis. Furthermore, these studies indicate that under these conditions lead-poisoned reticulocytes tend to accumulate nonhemoglobin iron on the cell membranes. Direct electron-microscopic evidence of iron accumulation in the stroma of immature red cells in lead poisoning has recently been reported by Bessis and Breton-Gorius (61). The finding here that blocking heme synthesis with lead caused an accumulation of iron in the reticulocyte membrane rather than in the water-soluble phase of the cell interior also indicates that, whereas the uptake of iron by the immature red cells may not be dependent upon heme synthesis, the release of iron from the membrane into the cell interior may be so dependent.

The failure of anoxia to stimulate iron uptake by reticulocytes *in vitro* (indeed its mild inhibitory effect) is in accord with observations of others that the maturation of immature red cells (62) and their incorporation of iron into heme (63) are somewhat inhibited, rather than stimulated, by anoxia. Thus, in terms of the ability of immature red cells to take up iron, to incorporate iron into heme and to mature morphologically, there is no evidence of a direct anoxic stimulus to red cell metabolism. The possibility that the rate of uptake of iron from IBP by reticulocytes *in vitro* is influenced by the presence of a plasma erythropoietic factor seems theoretically remote, since, in rabbits, this factor appears to act by stimulating cell division rather than the rate of maturation or hemoglobin metabolism (64). Indeed, it is characteristic of the active erythropoietic response to acute anoxia that the increase of red cells exceeds that of hemoglobin. Nevertheless, in an attempt to explore this possibility, the uptake by rabbit reticulocytes of Fe^{59} bound to rabbit plasma was measured in the presence of large volumes of plasma drawn before, and two days after, strenuous bleeding of the same rabbits. As determined by the Fe^{59} uptake and measurement of the iron content of the plasmas, there was no difference in the iron uptake by reticulocytes in the presence of "anemic" plasma as compared to "nonanemic" plasma.

To recapitulate the above, it is suggested that the utilization of iron by immature red cells can be separated into three phases: *1*) Iron bound to IBP is transferred by competitive binding to specific receptors on the cell surface. These receptors may be effaced by certain proteolytic enzymes. *2*) The functioning of these receptors is dependent upon metabolic energy, possibly for synthesizing receptors or for facilitating the release of iron from the receptors into the cell interior. This phase may be blocked by various inhibitors of oxidative metabolism. *3*) Iron is then incorporated into hemoglobin, presumably under the control of the microsomes and mitochondria of the cytoplasmic reticulum.[12] This stage may be blocked by low concentrations of lead.

It is of great interest that trivalent cobalt is bound by IBP in a manner comparable to iron. Cobalt, like iron, is capable of forming reversible combinations with molecular oxygen, when in the cobaltous state and linked to histidine (66). Its position in the porphyrin ring of the vitamin B_{12} molecule is analogous to that of iron in heme. Finally, it has the unique but still obscure capacity to stimulate erythropoiesis. It was found possible to substitute cobalt for iron in the interaction between IBP and the reticulocyte surface. However, the highly oxidant milieu needed for chelation of cobalt by IBP probably does not exist physiologically. Nor was it possible to produce polycythemia in rats repeatedly given cobalt combined with rat IBP *in vitro*. Finally, the fact that free Co^{++}, unlike iron and chromium, is itself taken up by immature red cells with greater avidity than by adult red cells may be more pertinent to the mechanism of cobalt polycythemia than the reactions between cobalt and IBP.

SUMMARY

Iron bound to purified human iron-binding protein (IBP) or to conalbumin, the iron-binding

[12] Although the nucleated red cells of birds have been widely employed in studies of heme synthesis, it has been clear for some time that the basophilic reticulum of the cytoplasm of immature red cells, not the nucleus, is the *sine quo non* of hemoglobin formation (65). As shown by Jensen and his associates (19) suspensions of avian red cells synthesize hemoglobin *in vitro* only in proportion to the concentration of reticulum-containing cells.

protein of egg white and of avian plasma, is readily and preferentially transferred to immature, but not to mature, mammalian red cells. Iron transfer is dependent upon the extent of saturation of IBP, and there exists a competition for iron between IBP and iron-binding receptors on the reticulocyte membrane. These cellular receptors are destroyed by certain proteolytic enzymes. Iron appears to be directly transferred to these receptors without existing in a free form; and iron once bound to reticulocyte membranes is not exchangeable with free iron and cannot be eluted with high concentrations of IBP or of ethylenediaminetetraacetic acid (EDTA).

The transfer of IBP-iron to immature red cells, unlike the adsorption of free iron, is related to metabolic energy-producing processes. Thus, it is accelerated by glucose and oxygen and is diminished or blocked by low concentrations of metabolic inhibitors, by nonphysiologic temperatures and by hemolysis. The uptake of iron is not primarily linked to heme synthesis. Appropriate concentrations of Pb^{++} blocked iron incorporation into heme by reticulocytes without reducing iron transfer, thus causing a pile-up of iron on the cell membranes.

Unlike reticulocytes, rat liver slices take up iron in appreciable amounts only at high IBP saturations. The transfer of iron from IBP to liver slices is not dependent upon metabolic energy and iron accumulated by liver slices can be eluted with unsaturated IBP.

Although free iron is usually bound equally by mature and immature red cells, free cobalt is more avidly bound by the immature cells. Trivalent cobalt, which forms colored chelates with purified IBP as does iron, also is bound by the IBP of whole plasma. Cobalt bound to IBP can, to a limited extent, be transferred preferentially to reticulocytes. However, the combination of Co^{+++} and IBP probably does not occur under physiologic conditions and is probably not involved in the induction of cobalt polycythemia.

REFERENCES

1. Farrant, J. L. An electron microscopic study of ferritin. Biochim. biophys. Acta 1954, **13**, 569.
2. Granick, S. Iron metabolism. Bull. N. Y. Acad. Med. 1954, **30**, 81.
3. Mazur, A., Baez, S., and Shorr, E. The mechanism of iron release from ferritin as related to its biological properties. J. biol. Chem. 1955, **213**, 147.
4. Holmberg, C. G., and Laurell, C. B. Investigations in serum copper. I. Nature of serum copper to the iron-binding protein in human serum. Acta chem. scand. 1947, **1**, 944.
5. Surgenor, D. M., Koechlin, B. A., and Strong, L. E. Chemical, clinical and immunological studies on the products of human plasma fractionation. XXXVII. The metal-combining globulin of human plasma. J. clin. Invest. 1949, **28**, 73.
6. Inman, J. K. Studies on human β_1-metal-combining protein. Thesis presented to the Division of Medical Sciences, Harvard University, 1956.
7. Schade, A. L., Reinhart, R. W., and Levy, H. Carbon dioxide and oxygen in complex formation with iron and siderophilin, the iron-binding component of human plasma (1, 2, 3). Arch. Biochem. 1949, **20**, 170.
8. Koechlin, B. A. Preparation and properties of serum and plasma proteins. XXVIII. The β_1-metal-combining protein of human plasma. J. Amer. chem. Soc. 1952, **74**, 2649.
9. Schade, A. L., and Caroline, L. Raw hen egg white and the role of iron in growth inhibition of Shigella dysenteriae, Staphylococcus aureus, Escherichia coli and Saccharomyces cerevisiae. Science 1944, **100**, 14.
10. Schade, A. L., and Caroline, L. An iron-binding component in human blood plasma. Science 1946, **104**, 340.
11. Warner, R. C., and Weber, I. The metal combining properties of conalbumin. J. Amer. chem. Soc. 1953, **75**, 5094.
12. Warner, R. C. The metal chelate compounds of proteins. Trans. N. Y. Acad. Sci. 1954, **16**, 182.
13. London, I. M., Shemin, D., and Rittenberg, D. The in vitro synthesis of heme in the human red blood cell of sickle cell anemia. J. biol. Chem. 1948, **173**, 797.
14. Shemin, D., London, I. M., and Rittenberg, D. The in vitro synthesis of heme from glycine by the nucleated red blood cell. J. biol. Chem. 1948, **173**, 799.
15. Walsh, R. J., Thomas, E. D., Chow, S. K., Fluharty, R. G., and Finch, C. A. Iron metabolism: Heme synthesis in vitro by immature erythrocytes. Science 1949, **110**, 396.
16. London, I. M., Shemin, D., and Rittenberg, D. Synthesis of heme in vitro by the immature nonnucleated mammalian erythrocyte. J. biol. Chem. 1950, **183**, 749.
17. Sharpe, L. M., Krishman, P. S., and Klein, J. R. Uptake by duck erythrocytes of iron added to blood. Arch. Biochem. 1952, **35**, 409.
18. London, I. M., and Yamasaki, M. Heme synthesis in non-intact mammalian and avian erythrocytes. Fed. Proc. 1952, **11**, 250.

19. Jensen, W. N., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. The uptake in vitro of radioactive iron by avian erythrocytes. J. Lab. clin. Med. 1953, **42**, 833.
20. Goldberg, A., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. Studies on the biosynthesis of heme in vitro by avian erythrocytes. Blood 1956, **11**, 821.
21. Bessis, M. C., and Breton-Gorius, J. Iron particles in normal erythroblasts and normal and pathological erythrocytes. J. biophys. biochem. Cytol. 1957, **3**, 503.
22. Bessis, M., and Breton-Gorius, J. Le cycle du fer dans l'organisme révélé par le microscope électronique. Sem. Hôp. Path. et Biol. 1957, No. XIX, 2173. (Ann. Rech. méd. 1957, No. 8, 829.)
23. Jandl, J. H., Inman, J. K., and Simmons, R. L. Transfer of iron and cobalt from serum iron-binding protein to human reticulocytes. Clin. Res. Proc. 1957, **5**, 144.
24. Inman, J. K. Preparation of crystalline beta,-metal-combining protein *in* Proc. 10th Conference on the Plasma Proteins and Cellular Elements of the Blood, Jan. 5–6, 1956, sponsored by the Protein Foundation, Inc., Cambridge, Mass., p. 46.
25. Bothwell, T. H., Hurtado, A. V., Donohue, D. M., and Finch, C. A. Erythrokinetics. IV. The plasma iron turnover as a measure of erythropoiesis. Blood 1957, **12**, 409.
26. Ham, T. H. A Syllabus of Laboratory Examinations in Clinical Diagnosis. Cambridge, Mass., Harvard University Press, 1950.
27. Rath, C. E., and Finch, C. A. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXXVIII. Serum iron transport. Measurement of iron binding capacity of serum in man. J. clin. Invest. 1949, **28**, 79.
28. Kitzes, G., Elvehjem, C. A., and Schuette, H. A. The determination of blood plasma iron. J. biol. Chem. 1944, **155**, 653.
29. Fischer, H. Hemin *in* Organic Synthesis, E. C. Horning, Ed. New York, John Wiley and Sons, Inc., 1955, coll. vol. 3, pp. 442–43.
30. Jandl, J. H., and Simmons, R. L. The agglutination and sensitization of red cells by metallic cations: Interactions between multivalent metals and the red-cell membrane. Brit. J. Haemat. 1957, **3**, 19.
31. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N. Preparation of lipide extracts from brain tissue. J. biol. Chem. 1951, **191**, 833.
32. Watson, C. J. The erythrocyte coproporphyrin. Arch. intern. Med. 1950, **86**, 797.
33. Eriksen, L. Lead intoxication. I. The effect of lead on the in vitro biosynthesis of heme and free erythrocyte porphyrins. Scand. J. clin. Lab. Invest. 1955, **7**, 80.
34. Klenk, E., and Lempfrid, H. Über die Natur der Zellreceptoren für das Influenza-virus. Z. physiol. Chem. 1957, **307**, 278.
35. Ada, G. L., and French, E. L. Purification of the receptor destroying enzyme of V. cholerae. Aust. J. Sci. 1950, **13**, 82.
36. Burnet, F. M., McCrea, J. F., and Stone, J. D. Modification of human red cells by virus action; receptor gradient for virus action in human red cells. Brit. J. exp. Path. 1946, **27**, 228.
37. Burnet, F. M. Principles of Animal Virology. New York, Academic Press Inc., 1955.
38. Borun, E. R., Figueroa, W. G., and Perry, S. M. The distribution of Fe^{59} tagged human erythrocytes in centrifuged specimens as a function of cell age. J. clin. Invest. 1957, **36**, 676.
39. Ellis, G. H., Brandt, C. S., and Thacker, E. J. Factors influencing the uptake of iron by blood and by bone marrow cells in vitro. Science 1954, **119**, 94.
40. Paoletti, C. Rôle des β-globulines plasmatiques dans le transport du fer utilisé par les cellules érythroformatrices. C. R. Acad. Sci. (Paris) 1957, **245**, 377.
41. Beutler, E. Iron enzymes in iron deficiency. I. Cytochrome C. Amer. J. med. Sci. 1957, **234**, 517.
42. Laurell, C. B. Studies on the transportation and metabolism of iron in the body with special reference to the iron-binding component in human plasma. Acta physiol. scand. 1947, **14**, Suppl. 46, 1.
43. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Thyroxine-binding by sera of pregnant women, newborn infants, and women with spontaneous abortion. J. clin. Invest. 1956, **35**, 1263.
44. Freinkel, N., Ingbar, S. H., and Dowling, J. T. The influence of extracellular thyroxine-binding protein upon the accumulation of thyroxine by tissue slices. J. clin. Invest. 1957, **36**, 25.
45. Gitlin, D., Janeway, C. A., and Farr, L. E. Studies on the metabolism of plasma proteins in the nephrotic syndrome. I. Albumin, γ-globulin and iron-binding globulin. J. clin. Invest. 1956, **35**, 44.
46. Yamakawa, T., and Suzuki, S. The chemistry of the lipids of posthemolytic residue or stroma of erythrocytes. II. On the structure of hemataminic acid. J. Biochem. (Tokyo) 1952, **39**, 175.
47. Gottschalk, A. Neuraminic acid; the functional group of some biologically active mucoproteins. Yale J. Biol. Med. 1956, **28**, 525.
48. Howe, C., Rose, H. M., and Schneider, L. Enzymic action of influenza virus on human erythrocyte stroma components. Proc. Soc. exp. Biol. (N. Y.) 1957, **96**, 88.
49. Tishkoff, G. H., Robscheit-Robbins, F. S., and Whipple, G. H. Red cell stroma in dogs. Variations in the stroma protein and lipid fractions related to experimental conditions. Blood 1953, **8**, 459.
50. Tishkoff, G. H. Personal communication.
51. Neilands, J. B. Some aspects of microbial iron metabolism. Bact. Rev. 1957, **21**, 101.
52. Hutner, S. H., Provasoli, L., Schatz, H., and Haskins, C. P. Some approaches to the study of the role

of metals in the metabolism of microorganisms. Proc. Amer. phil. Soc. 1950, **94**, 152.

53. Borsook, H., Abrams, A., and Lowy, P. H. Fructose-amino acids in liver: Stimuli of amino acid incorporation in vitro. J. biol. Chem. 1955, **215**, 111.
54. Kruh, J., and Borsook, H. Hemoglobin synthesis in rabbit reticulocytes in vitro. J. biol. Chem. 1956, **220**, 905.
55. Abrams, A., Lowy, P. H., and Borsook, H. Preparation of 1-amino-1-deoxy-2-keto hexoses from aldohexoses and α-amino acids. I. J. Amer. chem. Soc. 1955, **77**, 4794.
56. Glasstone, S. Textbook of Physical Chemistry, 2nd ed. New York, D. Van Nostrand Co., Inc., 1946.
57. Rapoport, S., and Hofmann, E. C. G. Untersuchungen über den Atmungsstoffwechsel von Reticulocyten. Biochem. Z. 1955, **326**, 493.
58. Schweiger, H. G., Rapoport, S., and Schölzel, E. Role of non-protein nitrogen in the synthesis of haemoglobin in the reticulocyte in vitro. Nature 1956, **178**, 141.
59. Hofmann, E. C., and Rapoport, S. Der Einfluss der Hämolyse auf die Atmung von Reticulocyten. Biochem. Z. 1955, **326**, 499.
60. Saltman, P., Fiskin, R. D., Bellinger, S. B., and Alex, T. The metabolism of iron by rat liver slices. The effect of chemical agents. J. biol. Chem. 1956, **220**, 751.
61. Bessis, M., and Breton-Gorius, J. Etude au microscope électronique du sang et des organes hémopoïétiques dans le saturnisme expérimental. Sem. Hôp. Path. et Biol. 1957, No. IV, 411. (Ann. Rech. méd. 1957, No. 2, 151.)
62. Rosin, A., and Rachmilewitz, M. Studies on bone marrow in vitro: III. The effect of anoxia and hyperoxia on explanted bone marrow. Blood 1948, **3**, 165.
63. Thomas, E. D. In vitro studies of erythropoiesis. II. The effect of anoxia on heme synthesis. Blood 1955, **10**, 612.
64. Erslev, A. J. The effect of anemic anoxia on the cellular development of nucleated red cells. Clin. Res. 1958, **6**, 187.
65. Reimann, F. Retikuläre Substanz und Hämoglobinbildung. Schweiz. Z. Path. 1943, **6**, 35.
66. Hearon, J. Z. The configuration of cobaltodihistidine and oxy-bis (cobaltodihistidine). J. nat. Cancer Inst. 1948, **9**, 1.

HYPERLIPEMIA IN EARLY STAGES OF ACUTE GLOMERULAR NEPHRITIS *

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Marked hyperlipemia, involving all lipid fractions, is a regularly noted observation in the nephrotic syndrome (1). In contradistinction, the serum lipid concentration is believed to be almost unaffected in early stages of acute glomerular nephritis, even though one can find data in the literature that indicate that increased concentrations of serum cholesterol may rarely be noted (1–5). As a consequence of continued interest in the pathogenesis of the nephrotic hyperlipemia (6) we have, during the past 10 years, obtained data on serum protein and/or albumin, cholesterol and /or total lipid concentration in 111 unselected cases of acute glomerular nephritis. The results of these studies will be presented and discussed in this communication. They indicate that the aforementioned contention can no longer be accepted without modification.

METHODS AND MATERIAL

Serum protein and albumin were determined according to the method of Weichselbaum (7). For the determination of total lipids and cholesterol in serum, the methods of Wilson and Hanner (8), and Schoenheimer and Sperry (9), respectively, were used. All samples were obtained after an overnight fast.

From 1948 to 1957, 248 children suffering from acute glomerular nephritis were admitted to Babies and Children's Hospital. The diagnosis was made on the basis of a preceding history of infection, the finding of proteinuria with hematuria, with or without fever, edema, hypertension and azotemia, and a benign course of the disease ending in cure. In 111 of these, chemical data were obtained which provide the material for our report. By chance, not by selection, neither cholesterol nor total lipid values were obtained at any time in the remaining 138 patients. No indication to obtain serum protein and lipid values prevailed except a well established diagnosis of acute glomerular nephritis, regardless of severity.

Nose and throat cultures were obtained in all but 11, and 31 of these yielded beta hemolytic streptococci. Three of these were typed [1] and were of the nephritogenic Type 12. Antistreptolysin "O" titers were obtained in 36 children. Thirty-one of these were greater than 333, the remaining five varied between 166 and 250. Forty-six children were re-examined more than four weeks after onset. Twenty-five of these were seen two to eight years after onset of the acute disorder. Urine examinations were negative in all. None of the remaining 65 children has been re-admitted with a relapse or with chronic renal disease. The age distribution of these 111 patients is charted in Table I. It is in agreement with previously established (1, 10) figures in that 75 per cent of the patients were older than four years of age, whereas in the nephrotic syndrome of unknown etiology the majority of the children are less than four years of age.

One hundred twenty-three children, 1 to 15 years of age, in whom either cholesterol, total lipids and/or serum protein and albumin values had been obtained, served as controls. Of these, 98 were free of infections. They had been admitted because of psychiatric, neurologic, allergic, orthopedic or ophthalmologic problems. In the 25 additional children of this group, blood was obtained during a febrile episode in 14, and 1 to 12 days after fever had subsided in 11. One of these patients had typhoid fever, two were febrile with rheumatoid arthritis and the re-

TABLE I

Age distribution of 111 children with acute glomerular nephritis

Age in years	No. of cases	Per cent
1	0	0
2	7	6.4
3	11	9.9
4	10	9.0
5	20	18.0
6	19	17.1
7	17	15.3
8	10	9.0
9	5	4.5
10	7	6.3
11	2	1.8
12	2	1.8
13	1	0.9

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[1] We are indebted to Dr. Floyd W. Denny from the Departments of Preventive Medicine and Pediatrics for carrying out the typing.

TABLE II

Distribution of serum protein and serum albumin values in children with acute glomerular nephritis and controls (with and without infections)*

	Acute glomerular nephritis		Controls			
			With infections		Without infections	
	No. of cases	Per cent	No. of cases	Per cent	No. of cases	Per cent
Serum protein						
Range of values	50		16		42	
Gm. %						
8–9	1	2	3	19	8	19
7–8	5	10	4	25	21	50
6–7	21	42	6	37	10	24
5–6	12	24	3	19	3	7
4–5	11	22	0	0	0	0
Serum albumin						
Range of values	34		16		37	
Gm. %						
5–6	1	3	0	0	21	57
4–5	9	26	12	75	13	35
3–4	16	47	3	19	3	8
2–3	7	21	1†	6	0	0
1–2	1	3	0	0	0	0

* First values obtained 6 to 24 days after onset of disease.

† Two and one-half year old male, typhoid fever. Value of 2.6 Gm. % obtained after three weeks of fever and poor intake of food.

maining 23 had upper respiratory infections of viral or bacterial origin. All were free of renal complications, in good state of nutrition, with the exception of the two and one-half year old patient with typhoid fever indicated in Table II.

RESULTS

Serum protein values were obtained in 50 and serum albumin values in 34 cases of acute glomerular nephritis. The first value, obtained 6 to 24 days after onset of the disease, is presented in Table II. These values can be compared with corresponding values obtained in 58 controls, 16 of whom had, or recently had had, one of the quoted infections. It can be seen that serum protein and albumin values below lowest values obtained in the controls were noted in over 20 per cent of the children with glomerular nephritis. More than one determination was obtained in 16 of these children. Figure 1 represents all values obtained in this group of children, and relates these values on the abscissa to the onset of the disease. It can be seen that hypoproteinemia and hypoalbuminemia were most marked 2 to 15 days after onset and persisted as long as 23 days after first symptoms of nephritis were observed.

Values for serum total lipids were obtained in 37 children with acute glomerular nephritis and 32 controls. Of the control subjects, 25 were free of any detectable infections, whereas seven had, or recently had suffered, a febrile illness. Table III shows that 43 per cent of the patients suffering from acute nephritis had on first measurement, 6 to 24 days after onset of the disease, higher total lipid values than were ever obtained in control subjects. A similar figure of 40 per cent is obtained from Table III as far as serum cholesterol concentration in the nephritic group of children is concerned. The statistical evaluation of these results yielded for total lipids as well as for cholesterol a value for p of < 0.01. More than one determination was obtained in 21 of these children. Figure 2 represents all values obtained in this group of children and relates the serum lipid and cholesterol concentration (ordinate) to the onset of the disease (abscissa). It may be seen that total lipid values may be increased as early as two days and cholesterol concentration as early as four days after onset. Highest values were obtained one to three weeks after the first symptoms of nephritis were noted. As a rule, initially elevated lipid and cholesterol values reached normal levels four weeks after onset. Repeated serial de-

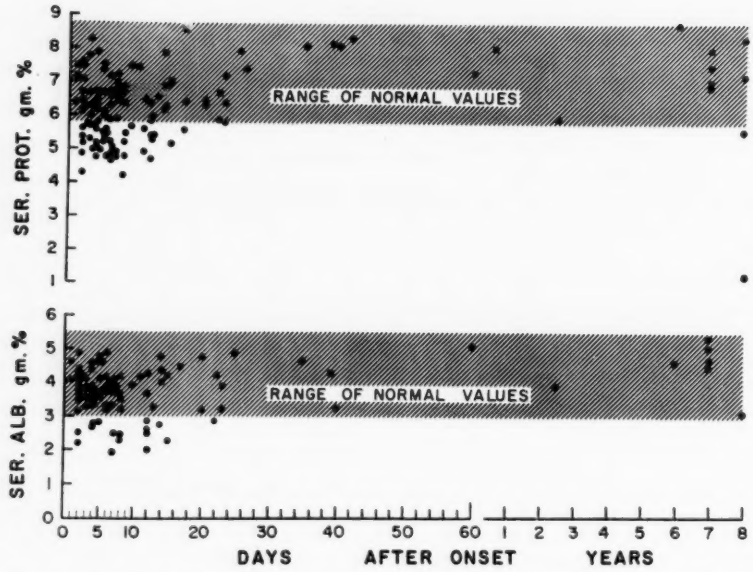

FIG. 1. SERUM PROTEIN AND SERUM ALBUMIN VALUES (GM. %) OBTAINED IN 50 AND 34 CHILDREN, RESPECTIVELY, WITH ACUTE GLOMERULAR NEPHRITIS

Shaded area represents values obtained in 58 and 53 control children, respectively.

TABLE III

Distribution of serum total lipid and cholesterol values in children with acute glomerular nephritis and controls (with and without infections)*

	Acute glomerular nephritis		Controls			
			With infections		Without infections	
	No. of cases	Per cent	No. of cases	Per cent	No. of cases	Per cent
Serum total lipids						
Range of values	37		7		25	
mg. %						
2,000–2,500	1	2.7	0	0	0	0
1,000–1,500	11	29.8	0	0	0	0
900–1,000	4	10.8	0	0	0	0
800– 900	4	10.8	1	14.3	1	4.0
700– 800	8	21.6	1	14.3	7	28.0
600– 700	8	21.6	3	42.8	7	28.0
500– 600	0	0	0	0	8	32.0
400– 500	0	0	1	14.3	2	8.0
300– 400	1	2.7	1	14.3	0	0
Serum cholesterol						
Range of values	55		29		84	
mg. %						
300– 400	8	14.5	0	0	0	0
260– 300	14	25.5	0	0	0	0
200– 260	16	29.0	3	10.2	19	22.65
150– 200	11	20.0	11	38.0	45	53.6
100– 150	6	11.0	11	38.0	19	22.6
50– 100	0	0	4	13.8	1	1.15

* First values obtained 6 to 24 days after onset of disease.

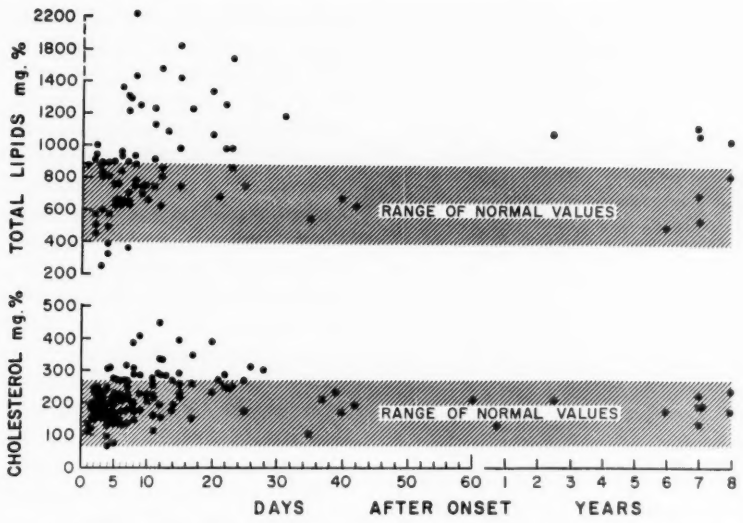

FIG. 2. TOTAL LIPID AND CHOLESTEROL VALUES IN SERUM (MG. %) OF 37 AND 55 CHILDREN, RESPECTIVELY, WITH ACUTE GLOMERULAR NEPHRITIS

Shaded area represents range of normal values obtained in 32 and 113 control children, respectively.

terminations were obtained in three patients and their values are reproduced in Table IV. It can be seen that in Patient No. 2 serum cholesterol concentration was still increased three months after onset, and that in Patient No. 1 cholesterol and total lipid values had not returned to normal values eight months after onset even though urine examination did not reveal any abnormality at this time. In Patient No. 1 total lipid values were still markedly elevated one month after onset. It is worthy of note that these increased values for serum lipids were obtained at a time when serum protein and albumin values had returned to normal three to six months previously.

Table V lists the distribution, mean and median values for serum cholesterol and total lipid con-

TABLE IV

Behavior of serum protein, serum albumin, serum cholesterol and total lipids in three children with acute glomerular nephritis followed up to eight months after onset

	Weeks after onset				Months after onset				
	1	2	3	4	2	3	5	6	8
Patient No. 1									
Serum protein (*Gm. %*)	4.6	6.7			6.2		7.0		7.6
Serum albumin (*Gm. %*)	2.4	3.6					4.5		5.0
Cholesterol (*mg. %*)	382	420			375		296		270
Total lipids (*mg. %*)	1,480				1,180		1,350		900
Patient No. 2									
Serum protein (*Gm. %*)		5.2	6.0	6.9		7.2		7.7	
Serum albumin (*Gm. %*)		2.6	3.4			3.7		5.5	
Cholesterol (*mg. %*)		270	270	290		345		230	
Total lipids (*mg. %*)		1,250	960	1,100		550		710	
Patient No. 3									
Serum protein (*Gm. %*)	4.9	5.3	6.3						8.6
Serum albumin (*Gm. %*)	1.9	2.4	3.1						4.6
Cholesterol (*mg. %*)	318	392	391						223
Total lipids (*mg. %*)	1,210	1,580	1,340	1,680					710

TABLE V

Bacteriologically and/or serologically confirmed cases of acute glomerular nephritis

	Acute glomerular nephritis		Controls	
	No. of cases	Per cent	No. of cases	Per cent
Serum total lipids				
Range of values	37		32	
mg. %				
300– 400	2	5.4	0	0
400– 500	0	0	3	9.4
500– 600	4	10.8	9	28.1
600– 700	3	8.1	10	31.3
700– 800	5	13.5	8	25.0
800– 900	6	16.2	1	3.1
900–1,100	8	21.7	1	3.1
1,100–1,500	6	16.2	0	0
1,500–2,000	2	5.4	0	0
2,000–2,500	1	2.7	0	0
Average (*mg. %*)	921		629	
Median (*mg. %*)	857		634	
Serum cholesterol				
Range of values	47		92	
mg. %				
90– 100	1	2.12	1	1.1
100– 150	7	14.9	21	22.7
150– 200	14	29.8	48	52.2
200– 250	11	23.4	20	21.8
250– 300	10	21.2	2	2.2
300– 350	2	4.25	0	0
350– 400	0	0	0	0
400– 450	2	4.25	0	0
Average (*mg. %*)	216		172	
Median (*mg. %*)	205		175	

centration obtained in 47 to 37 cases, respectively, in whom the diagnosis of acute glomerular nephritis was supported by positive throat cultures for beta hemolytic streptococci or by antistreptolysin titers greater than 333. A statistical evaluation of this material reveals that total lipid as well as cholesterol values were more frequently higher than normal in the serum of the nephritic patients ($p < 0.01$).

DISCUSSION

It should be pointed out that the 111 cases of glomerular nephritis in whom serum protein and/or albumin, cholesterol and/or total lipid values were obtained represented a group of unselected cases of acute glomerular nephritis.

Doubt could arise as to whether or not the diagnosis of acute glomerular nephritis was correct in every case studied. The usual criteria for the diagnosis were used as outlined before. None of the cases used in this study has to our knowledge relapsed or shown evidence of chronic renal disease. Bacteriological and serological tests obtained in support of the diagnosis and re-examinations performed have been previously mentioned. From the 39 patients with acute glomerular nephritis that had increased cholesterol or total lipid values during the acute phase of the disease, 26 (67 per cent) were re-examined more than two months after onset of the disease. Twelve were seen two months to one year after onset, and 14, one to nine years after onset. Negative urine examinations, normal blood pressure readings, blood urea nitrogen (BUN) values and red blood cell sedimentation rates were obtained in all. If doubts were to be maintained as to whether or not a case of nephrosis may have been included in our series, it should be pointed out that statistically significantly higher serum cholesterol and total lipid values were also obtained in the 47 cases of nephritis that were bacteriologically or serologically supported by either positive throat cultures for beta hemolytic streptococci or antistreptolysin "O" titers greater than 333.

Hypoproteinemia and hypoalbuminemia in early phases of glomerulonephritis have been described by others prior to this study (11–13). The degree of proteinuria in acute glomerular nephritis is usually not severe enough to explain satisfactorily the depletion of plasma proteins. Hydremia has been noted in the edematous stage of this disorder (12, 14). At least in part, this may play a role in the pathogenesis of hypoproteinemia and hypoalbuminemia of acute glomerular nephritis.

We believe that our studies have shown that a mild to moderate degree of hyperlipemia may be noted in 40 to 43 per cent of children suffering from acute glomerular nephritis. The finding of moderately increased serum cholesterol or total lipid values thus no longer should exclude the diagnosis of acute glomerular nephritis. Hyperlipemia is almost invariable in children with the nephrotic syndrome.[2]

[2] At a recent "Yearly Conference on the Nephrotic Syndrome" the participants were asked whether they had ever seen a child in the active phase of the disease without hyperlipemia. None of them had. The combined experience of these participants must have comprised more than 500 cases. In our own experience with 219

It has been proposed (15) that in the nephrotic syndrome hypoalbuminemia plays a causal role in the elicitation and maintenance of the nephrotic hyperlipemia. An evaluation of the data presented in this communication, obtained in children suffering from acute glomerular nephritis, reveals that of 35 total lipid values that were above normal range, 12 were obtained in children in whom serum albumin values were lower than normal, whereas 23 (65 per cent) were noted in children with normal serum albumin values. Of 16 hypercholesteremic values, nine had hypoalbuminemia whereas the remaining seven (or 44 per cent) were obtained in sera with albumin concentrations within normal range. Considering further that the nephritic hypoalbuminemia may be due to a great extent to hydremia rather than to urinary losses of albumin, we doubt that our data support the hypothesis (15) of a causal relationship between hypoalbuminemia and hyperlipemia.

Both hypoalbuminemia and hyperlipemia, whenever noted in acute glomeruler nephritis, are less severe than in the nephrotic syndrome. If, however, hypoproteinemia and hyperlipemia may occur in early stages of acute glomerular nephritis, and if for a few days proteinuria, edema and ascites may in some of these children be marked, a mild and transient nephrotic syndrome then may be noted. Six of our 111 cases presented this picture. Inasmuch as hematuria no longer serves as a valuable aid in the differential diagnosis between nephritis and nephrosis (1, 10, 16, 17), other procedures have to be resorted to. Bacteriological and serological tests have proved their value in supporting the diagnosis of glomerular nephritis from an etiological point of view. Nose and throat cultures are of obvious importance if they yield beta hemolytic streptococci (18). The type specificity of these organisms for nephritogenic strains can be determined and can be most helpful. Antistreptolysin "O" titers yield low values in the nephrotic syndrome and are usually greater than 333 in acute glomerular nephritis. In single instances one might have to wait for the course of the disease to decide the diagnostic problem. In acute glomerular nephritis a benign course would be expected, while in nephrotic children with hematuria a benign course with rapid clearing would be most unusual.

children suffering from the nephrotic syndrome and seen during the last 26 years at Babies and Children's Hospital, hypercholesteremia and/or hyperlipemia was noted in every instance.

SUMMARY

1) In 111 children suffering from acute glomerular nephritis, serum protein, albumin, cholesterol and total lipid values were obtained during early stages of the disease. These were compared with corresponding values obtained in 123 controls of similar age. Twenty-three of these had recent infections, while 98 had been free of infection and were free of any condition that conceivably may have an effect on serum protein or lipid concentration.

2) Lower values for serum protein and albumin concentration than any obtained in the controls were noted in 20 per cent of the nephritic group. In these, hypoproteinemia and albuminemia were most marked 2 to 15 days after onset and persisted for as long as 23 days.

3) Higher values for serum cholesterol and total lipid concentration than any obtained in the control group were noted in 40 per cent and 43 per cent, respectively, of the children with acute glomerular nephritis. This mild to moderately severe hyperlipemia may be observed as early as two to four days after onset and the highest values were obtained one to three weeks after first symptoms of the disease had been noted. A return to normal levels was usually observed after four weeks, though some took as long as three to eight months to reach the normal range.

4) The presence of mild to moderately severe hyperlipemia should no longer be considered to exclude acute glomerular nephritis. Bacteriological and serological confirmation of the diagnosis of glomerular nephritis by obtaining nose and throat cultures and antistreptolysin "O" titers is recommended.

REFERENCES

1. Barnett, H. L., Forman, C. W., and Lauson, H. D. The nephrotic syndrome in children. Advanc. Pediat. 1952, **5**, 81, 99.
2. Fishberg, A. M. Hypertension and Nephritis, 4th ed. Philadelphia, Lea & Febiger, 1944, p. 473.
3. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry. Vol. I, Interpretations, 2nd ed. Baltimore, Williams and Wilkins, 1946, p. 531.

4. Maxwell, J. A further note on the plasma cholesterol in nephritis. Quart. J. Med. 1934, **3**, 79.
5. Peters, J. P., and Man, E. B. The interrelations of serum lipids in patients with diseases of the kidneys. J. clin. Invest. 1943, **22**, 721.
6. Heymann, W., Nash, G., Gilkey, C., and Lewis, M. Studies on the causal role of hypoalbuminemia in experimental nephrotic hyperlipemia. J. clin. Invest. 1958, **37**, 808.
7. Weichselbaum, T. E. Accurate and rapid method for determination of proteins in small amounts of blood serum and plasma. Amer. J. clin. Path. 1946, **10**, 40.
8. Wilson, W. R., and Hanner, J. P. Changes of total lipid and iodine number of blood fat in alimentary lipemia. J. biol. Chem. 1934, **106**, 323.
9. Schoenheimer, R., and Sperry, W. M. A micromethod for the determination of free and combined cholesterol. J. biol. Chem. 1934, **106**, 745.
10. Heymann, W., and Alperin, L. J. Lipemic nephrosis with and without nephritis. Amer. Practit. 1949, **3**, 680.
11. Addis, T. Glomerular Nephritis. New York, The Macmillan Company, 1949, p. 194.
12. Cardozo, E. L. Hydremia in acute nephritis. Acta med. scand. 1946, **125**, 333.
13. Rubin, M. I. Disturbances of the kidney *in* Textbook of Pediatrics, W. E. Nelson, Ed., 5th ed. Philadelphia, W. B. Saunders Company, 1950, p. 1212.
14. Earle, D. P., and Seegal, D. Natural history of glomerulonephritis. J. chron. Dis. 1957, **5**, 3.
15. Rosenman, R. H., Friedman, M., and Byers, S. O. The causal role of plasma albumin deficiency in experimental nephrotic hyperlipemia and hypocholesteremia. J. clin. Invest. 1956, **35**, 522.
16. Barness, L. A., Moll, G. H., and Janeway, C. A. Nephrotic syndrome; natural history of the disease. Pediatrics 1950, **5**, 486.
17. Heymann, W., Rothenberg, M. B., Gilkey, C., and Lewis, M. Difference in color of hematuria in the nephrotic syndrome and glomerulonephritis. Pediatrics 1958, **21**, 375.
18. Rammelkamp, C. H., Jr. Microbiologic aspects of glomerulonephritis. J. chron. Dis. 1957, **5**, 28.

SODIUM AND WATER DEPLETION AND AMMONIA FORMATION BY THE KIDNEYS *

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(Submitted for publication July 14, 1958; accepted September 25, 1958)

Gamble and associates' work on the mechanism of action of acidifying salts (1, 2) originated the impression that the increase in renal excretion of ammonia in response to acidosis of several days' duration might be related to the loss of sodium and water induced by the first doses of the drug (3). This impression has never been adequately substantiated or disproven over the last three decades. In 1952 Peters (4) thus summarized his views on this matter: "The precise stimulus for the secretion of ammonia has not yet been identified. . . . The impression is given that it is substituted for sodium only after a certain amount of this base has been sacrificed and continues to be secreted until the normal supply of sodium in the body has been restored."

Pitts (5) admitted the same possibility while postulating that an increased secretion of adrenal cortical hormones, stimulated by sodium depletion, was the mechanism whereby ammonia excretion increased when ammonium chloride was given to rats.

The degree of acidosis does not seem to be the main regulating influence (6) because in Gamble's experiments ammonia excretion continues to increase even after the acidosis has reached its maximum, and maximal values of ammonia excretion occur while extracellular acidosis is being reversed. Many years ago Briggs (7, 8) expressed the opinion that renal excretion of ammonia had nothing to do with regulation of acid-base balance of the "milieu interieur," and that it served the only purpose of avoiding the harmful action of an excessively acid urine on the walls of the urinary tract. His views were based on experiments in which sodium sulfate solution was infused into dogs with subsequent increase in the excretion of ammonia in a urine of low pH, although there was no sodium or water depletion. Comparable findings had been reported by Hendrix and Sanders (9), and were recently demonstrated by Schwartz, Jenson and Relman (10).

However, all those experiments in which an increase in ammonia excretion could be demonstrated in the absence of sodium and water depletion were of short duration (minutes to hours). It is very likely that this immediate increase in ammonia excretion results from the passage of preformed ammonia from within the tubular cells into the tubular lumen (11). The slow and stepwise increase in ammonia excretion over the period of several days, such as was seen in Gamble's experiments and subsequently confirmed by many others, seems to result from an intensified activity of ammonia-forming enzymes of the tubular cells (12), and might well be controlled by factors different from those which determine the release of preformed ammonia.

The following experiments were planned to investigate whether the depletion of sodium and water which follows the ingestion of acidifying salts is an indispensable factor in determining the coincident increase in urinary ammonia excretion.

Our results demonstrate that even in the absence of depletion of the subjects' own reserves of sodium and water, acidosis induced by ammonium chloride ingestion is accompanied by a stepwise increase in urinary ammonia, comparable to that seen in the experiments of Gamble, Blackfan and Hamilton (2) and of Sartorius, Roemmelt and Pitts (13). Although the increase in ammonia excretion does contribute to the economy of fixed cation, it is not the state of water and sodium depletion which constitutes the stimulating agent for the increase in activity of ammonia-forming enzymes of the renal tubular cells.

* This paper is based on a thesis accepted as part of the requirement for the Professorship of Medicine at the University of Bahia, Bahia, Brasil. The work was aided by a grant from the Conselho Nacional de Pesquisas (National Research Council), Brasil.

TABLE I

Effect of ammonium chloride on electrolyte metabolism

Day	Body weight*	Urine							Plasma*	Serum*		
		Vol.	pH	Ammonia	Titr. acid	Cl^-	Na^+	K^+	Total CO_2	Cl^-	Na^+	K^+
	Kg.	*ml./day*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
1	56.00	1,620	7.10	24.29	8.75	328.2	392.8	64.8	25.4	102	140	4.1
2	57.80	2,920	7.03	39.71	16.64	663.1	695.0	77.7				
3	57.65	3,660	6.84	41.90	16.84	675.6	702.7	71.7				
4†	57.25	3,280	6.52	48.50	27.22		705.2	75.4	26.1	104	142	4.5
5	57.35	4,100	5.10	102.32	56.58	947.9	820.0	96.4	24.0	109	142	4.5
6	56.65	3,480	5.01	120.56	60.20	741.9	631.6	94.0	22.5	111	148	4.2
7	56.35	3,210	5.12	133.08	57.14	808.3	680.5	86.7	18.6	117		4.2
8	56.45								22.5		143	4.2

* Values in these columns were obtained at the beginning of each experimental day.
† 10 Gm. of ammonium chloride was administered orally on each day starting on the fourth day.

MATERIAL AND METHODS

Five healthy adults, aged 22 to 29 years, were maintained on a constant and adequate diet for a period of seven days, during which they ingested 2.5 L. of a solution containing 9 Gm. per L. of sodium chloride and 2.3 Gm. per L. of sodium bicarbonate. In addition, the subjects drank a constant amount of distilled water, which was always more than 1 L. per day.

The first three days of each experiment were taken as controls. From the fourth through the seventh day of each experiment, while still on the same diet and drinking the same amount of the above described solution, the subjects were given orally 10 Gm. of ammonium chloride daily, in four equally divided doses.

The urine was carefully collected under toluene for 24 hour periods, and on each specimen the following determinations were done: *1*) volume per 24 hours, expressed in milliliters; *2*) pH, measured with a "Cambridge" pH meter, Research Model; *3*) titratable acidity, by Henderson and Palmer's method, as described by Consolazio, Johnson and Marek (14); *4*) ammonia, by Conway's method (15); *5*) chlorides, by Van Slyke and Hiller's method, as described by Hawk, Bernard and Summerson (16); *6*) sodium, measured with a "Baird" flame photometer, using an internal standard of lithium sulfate; and *7*) potassium, same as for sodium. Values referring to concentration of ammonium, titratable acidity, chloride, sodium and potassium are expressed in mEq. per day.

Blood samples were taken at the beginning of Days 1, 4, 5, 6 and 7, and by the end of the seventh metabolic day. All specimens were taken from the veins of the metacarpal region after immersing the forearm in water at 46° C. for 20 minutes. One aliquot of blood was handled under anaerobic conditions and mixed with heparin. On the plasma separated from that blood, total carbon dioxide content was measured in a manometric Van Slyke apparatus. The rest of the blood was allowed to clot in contact with air in another test tube. On the serum separated therefrom, chloride, sodium and potassium concentrations were measured. The methods were the same as for the respective urine determinations.

TABLE II

Effect of ammonium chloride on electrolyte metabolism

Day	Body weight*	Urine							Plasma*	Serum*		
		Vol.	pH	Ammonia	Titr. acid	Cl^-	Na^+	K^+	Total CO_2	Cl^-	Na^+	K^+
	Kg.	*ml./day*		*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./day*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
1	72.25	1,480	6.84	40.75	10.51	479.8	509.1	88.8	24.6	104	146	4.2
2	73.85	2,367	7.10	39.69	9.23	562.9	601.2	60.6				
3	74.00	3,460	6.93	46.03	18.68	716.2	751.5	65.0				
4†	73.50	4,100	6.04	56.09	30.34	936.0	910.2	70.5	28.5		150	4.5
5	72.30	2,550	5.25	62.78	43.09	780.3	772.6	72.7		115	147	4.9
6	72.30	3,150	4.98	105.80	48.51	827.5	745.0	85.0	19.8	112	146	4.6
7	71.95	3,360	5.05	126.64	49.06	794.3	756.0	90.7	18.4	112	146	4.5
8	71.90								19.2	113	145	4.4

* Values in these columns were obtained at the beginning of each experimental day.
† 10 Gm. of ammonium chloride was administered orally on each day starting on the fourth day.

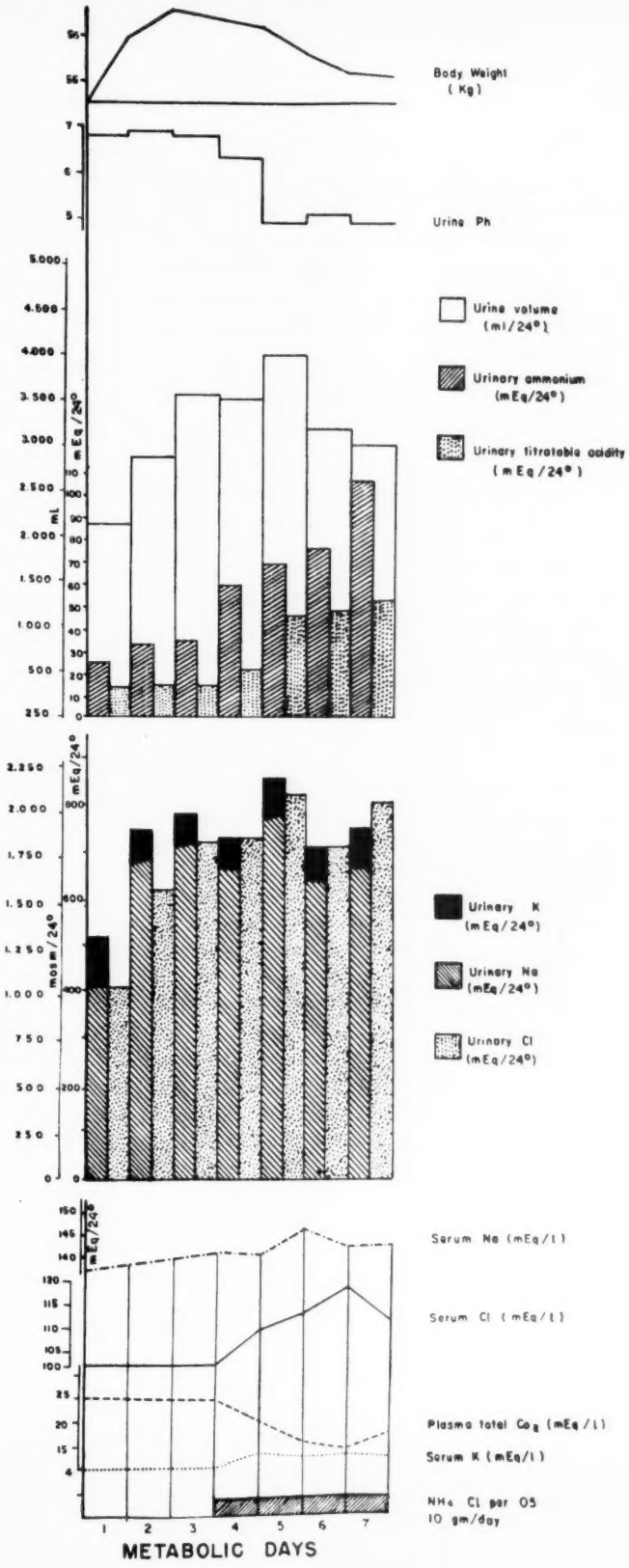

FIG. 1. EFFECT OF AMMONIUM CHLORIDE ON ELECTROLYTE BALANCE

During the first three days of the study, body water and sodium content were expanded by repeated ingestion of a solution containing sodium chloride and sodium bicarbonate. The ingestion of ammonium chloride starting on the fourth experimental day resulted in loss of body weight which, however, did not fall beyond control levels. In spite of the absence of depletion of the body's own stores of sodium and water, the progressive stepwise increase in urinary ammonia was observed.

RESULTS

The results of typical experiments are summarized in Figure 1 and in Tables I and II. None of the subjects complained of any untoward reactions to the experimental regimen. Throughout the experiments, they were capable of carrying on their routine activities in medical wards and laboratories.

Figure 1, for one of the typical experiments, shows how the body weight increased during the control days as a result of the ingestion of sodium chloride and bicarbonate solution. Ingestion of ammonium chloride starting on the fourth experimental day, with ensuing acidosis, caused a decrease in body weight which, however, did not reach precontrol levels. The subject did not sacrifice his own reserves of sodium and water. Ammonia excretion, which had been approximately constant during the control period, increased progressively in a stepwise fashion to levels comparable to those reported by others (2, 13) under conditions of progressive sodium and water depletion.

Results comparable to those described above were also found in two other experiments, one of which is shown in Table I. Two other subjects were also submitted to the same experimental regimen, but their body weights fell to less than precontrol levels (see Table II) during administration of ammonium chloride. They, however, showed an increase in urinary ammonia excretion comparable to that found in the three subjects who had obviously not been depleted of their own reserves of sodium and water.

Control levels of urinary pH were high, and those of titratable acidity were low in all experiments consequent to the ingestion of sodium bicarbonate. As would be expected, urinary pH decreased and the urinary excretion of chloride and titratable acid increased in all experiments while ammonium chloride was being administered.

The ingestion of ammonium chloride resulted in a marked increase in sodium and in total solutes excretion over and above control levels which were already high. However, we did not find a progressive decrease in the urinary excretion of sodium over several days, such as Gamble and coworkers (2) and Sartorius and associates (13) described in subjects who were being depleted of sodium and water. Nor was the increase in potassium excretion following ammonium chloride administration of such magnitude as that found in Sartorius' experiments.

The total carbon dioxide content of the arterial plasma increased during the control days, due to the ingestion of sodium bicarbonate, and decreased when ammonium chloride was administered. By the fourth day of acidosis, the carbon dioxide content of the plasma was increasing while ammonium chloride was still being given. Serum chloride values were high in all experiments during the control periods and increased further when ammonium chloride was administered. Serum sodium concentration increased during the control days and showed a slight and irregular decrease while the subjects were on ammonium chloride. No regular pattern could be made out of the variations in serum potassium concentration.

DISCUSSION

Gamble and associates demonstrated that the excretion of fixed cation is maximal on the first day of administration of an acidifying salt and decreases progressively on the following days, while ammonia excretion increases (2). The initial loss of fixed cation is associated with high urinary volume and loss of weight. After discontinuing the acidifying salt, a positive sodium balance occurs, and in spite of elevation of urinary pH, urinary ammonia excretion continues at high levels. This was thought to last until the body regained its stores of fixed cations and water.

Our results demonstrate the same characteristic stepwise increase in ammonia excretion consequent to the ingestion of an acidifying salt over several days, even when the depletion of sodium and water was avoided.

Various types of experiments, some of which were published long ago, have shown that urinary ammonia excretion can increase in the absence of sodium and water depletion. This happens, for instance, when normal human subjects breathe gas mixtures containing carbon dioxide in a concentration of 5 to 6 per cent (17), as well as after infusion of sodium salts of nonreabsorbable anions (8–10). In all those experiments urinary pH was suddenly brought to very low levels.

Several lines of evidence suggest that renal

excretion of ammonia can be modified according to two entirely different patterns. Sudden changes in urinary pH are promptly accompanied by the *release* of larger or smaller amounts of ammonia, which very likely were previously formed and present inside the tubular cells (11). On the other hand, conditions prevailing inside the tubular cells seem to determine a faster or slower rate of *formation* of ammonia (12). Thus, a drop in urinary ammonia was found to occur simultaneously with an increase in urinary pH caused by carbonic anhydrase inhibitors (18), while the prolonged use of carbonic anhydrase inhibitors, with ensuing acidosis, was found to cause increase in urinary ammonia excretion (19). This difference in patterns of change in urinary ammonia excretion is further illustrated by data reported by Sartorius (13), who measured the immediate change in ammonia excretion after administration of ammonium chloride to normal humans and found an increase of several fold within the first two hours. This rate was obviously not maintained throughout the several days during which ammonium chloride was given. It is likely that this large initial increase was due to transfer of preformed ammonia from within the tubular cells to the tubular lumen, as a result of sudden acidification of the urine. The progressive, but much slower, increase of several days' duration would result from the intensified action of ammonia-forming enzymes under the stimulus of acidosis.

These two patterns of change in urinary ammonia excretion seem to be influenced by factors of a different order. Increased ammonia excretion in the absence of sodium and water depletion had been found during experiments of a few hours' duration. This did not rule out the possibility that the same factor of sodium and water depletion might be the stimulus for the stepwise progressive increase in ammonia excretion consequent to the administration of acidifying salts for several days.

In our experiments ammonium chloride was given to normal human subjects preloaded with sodium chloride and bicarbonate. Sodium bicarbonate was added in order to avoid the acidifying effect of large amounts of isotonic sodium chloride solution given prior to the administration of ammonium chloride. Administration of ammonium chloride from the fourth to the seventh experimental days resulted in loss of some of the surplus of water and fixed cation, but in three out of the five experiments, body weight did not reach precontrol levels during the experimental period. These subjects excreted the excess chloride ingested as ammonium chloride along with the fixed cation and the water accumulated during the control days, and did not have to sacrifice their own reserves of sodium and water. The progressive decrease in sodium excretion, which in the experiments of Gamble and of Sartorius indicated the need for the body to save fixed cation, was not present in our experiments. In spite of that, the daily increase in ammonia excretion proceeded at a rate comparable to that described by Gamble (2), and subsequently confirmed by many others (13, 20, 21), under conditions in which water and sodium depletion did take place. No significant difference in the pattern of ammonia excretion could be detected amongst our own subjects, between those whose body weight remained above precontrol levels throughout the experiment and those who lost some of their own reserves of sodium and water.

The factors that control urinary excretion of ammonia have not yet been fully described. It was already pointed out that in Gamble's experiments the increase in ammonia excretion and the extracellular acidosis failed to follow parallel courses. This can also be seen in the data herein reported, and points to the existence of other factors which determine the progressive and stepwise increase in urinary ammonia excretion. The latter part of Gamble's experiments (2) indicates that urinary ammonia excretion can still be high in long-term experiments in spite of elevation of urinary pH. The increase in excretion of potassium following ammonium chloride administration in our experiments was irregular and small, much less than in Sartorius's experiments. This indicates that potassium depletion in itself should not be considered a prerequisite for the increase in ammonia excretion in response to acidifying salts.

As shown by Davies and Yudkin (22), adaptation of ammonia-forming enzymes of the renal tubular cells to a low pH proceeds at a slow rate. Schwartz, Jenson and Relman (10, 23) suggested that the progressive loss of cation during the first days of administration of the acidifying salt

would aggravate the difference between the excretion of fixed anions and the fixed cations available for excretion. This would stimulate ammonia formation in a stepwise manner.

CONCLUSIONS

1. Electrolyte and acid-base studies were made on five normal subjects to determine if cation depletion is essential for progressive stepwise increase in urinary ammonia excretion secondary to administration of acidifying salts.

2. Subjects were maintained on constant diet and fluid intake and given sufficient sodium chloride and sodium bicarbonate orally to expand the extracellular fluid by approximately 1.5 to 2 L.

3. When extracellular fluid volume was increased, 10 Gm. of ammonium chloride was administered daily for four days. This resulted in a progressive excretion of ammonia although body weight measurements indicated that the extracellular fluid volume was still excessive.

4. These results demonstrate that depletion of sodium is not a prerequisite for a sustained increase in urinary ammonia excretion.

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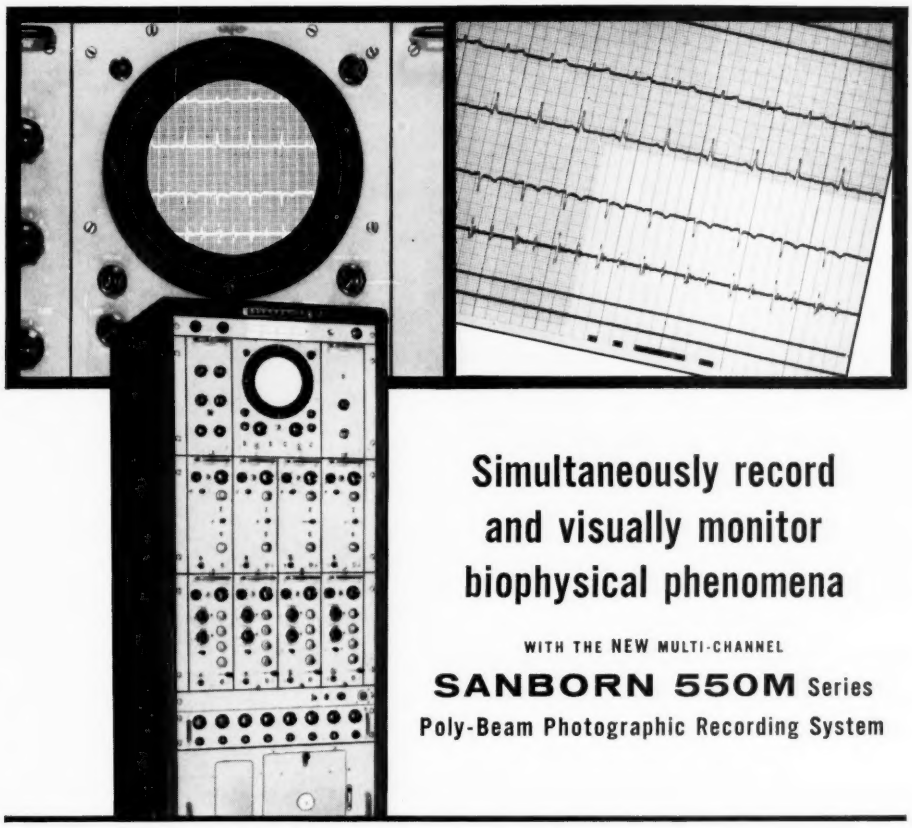
REFERENCES

1. Gamble, J. L., Ross, G. S., and Tisdall, F. F. Studies of tetany. I. The effect of calcium chloride ingestion on the acid-base metabolism of infants. Amer. J. Dis. Child. 1923, **25**, 455.
2. Gamble, J. L., Blackfan, K. D., and Hamilton, B. A study of the diuretic action of acid producing salts. J. clin. Invest. 1925, **1**, 359.
3. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry. Vol. 1, Interpretations. Baltimore, Williams & Wilkins Co., 1932.
4. Peters, J. P. Water balance in health and [illegible]ease in Diseases of Metabolism, 3rd ed., G. G. D[illegible]an, Ed. Philadelphia, W. B. Saunders Co., 1952, p. 315.
5. Pitts, R. F. Acid-base regulation by the kidneys. Amer. J. Med. 1950, **9**, 356.
6. Folling, A. On the mechanism of the ammonium chloride acidosis. Acta med. scand. 1929, **71**, 221.
7. Briggs, A. P. Excretion of ammonia and neutrality regulation. J. biol. Chem. 1934, **104**, 231.
8. Briggs, A. P. The significance of the urinary ammonia. J. Lab. clin. Med. 1942, **28**, 174.
9. Hendrix, B. M., and Sanders, J. P. The effect of injections of sodium phosphates and sodium hippurate upon the excretion of acid and ammonia by the kidney. J. biol. Chem. 1923, **58**, 503.
10. Schwartz, W. B., Jenson, R. L., and Relman, A. S. Acidification of the urine and increased ammonium excretion without change in acid-base equilibrium: Sodium reabsorption as a stimulus to the acidifying process. J. clin. Invest. 1955, **34**, 673.
11. Clarke, E., Evans, B. M., MacIntyre, I., and Milne, M. D. Acidosis in experimental electrolyte depletion. Clin. Sci. 1955, **14**, 421.
12. Rector, F. C., Jr., Seldin, D. W., and Copenhaver, J. H. The mechanism of ammonia excretion during ammonium chloride acidosis. J. clin. Invest. 1955, **34**, 20.
13. Sartorius, O. W., Roemmelt, J. C., and Pitts, R. F. The renal regulation of acid-base balance in man. IV. The nature of the renal compensations in ammonium chloride acidosis. J. clin. Invest. 1949, **28**, 423.
14. Consolazio, C. F., Johnson, R. E., and Marek, E. Metabolic Methods; Clinical Procedure in the Study of Metabolic Functions. St. Louis, C. V. Mosby Co., 1951.
15. Conway, E. J. Micro-diffusion Analysis and Volumetric Error, 3rd ed. rev. London, Lockwood, 1950.
16. Hawk, P. B., Bernard, L. O., and Summerson, W. H. Practical Physiological Chemistry, 13th ed. New York, Blakiston Co., 1954.
17. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L. Experiments on the regulation of the blood's alkalinity. I. J. Physiol. 1920, **54**, 32.
18. Ferguson, E. B., Jr. A study of the regulation of the rate of urinary ammonia excretion in the rat. J. Physiol. 1951, **112**, 420.
19. Rector, F. C., Jr., Seldin, D. W., Roberts, A. D., Jr., and Copenhaver, J. H. Relation of ammonia excretion to urine pH. Amer. J. Physiol. 1954, **179**, 353.
20. Ryberg, C. On the formation of ammonia in the kidney during acidosis. Acta physiol. scand. 1948, **15**, 114.
21. Wood, F. J. Ammonium chloride acidosis. Clin. Sci. 1955, **14**, 81.
22. Davies, B. M. A., and Yudkin, J. Studies in biochemical adaptation. The origin of urinary ammonia as indicated by the effect of chronic acidosis and alkalosis on some renal enzymes in the rat. Biochem. J. 1952, **52**, 407.
23. Schwartz, W. B., Jenson, R. L., and Relman, A. S. The disposition of acid administered to sodium-depleted subjects: The renal response and the role of whole body buffers. J. clin. Invest. 1954, **33**, 587.



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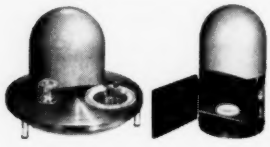

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STUDIES ON INFLUENZA IN THE PANDEMIC OF 1957–1958. I. AN EPIDEMIOLOGIC, CLINICAL AND SEROLOGIC INVESTIGATION OF AN INTRAHOSPITAL EPIDEMIC, WITH A NOTE ON VACCINATION EFFICACY *

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(Submitted for publication July 10, 1958; accepted August 7, 1958)

Influenza caused by a new variant of influenza A virus assumed epidemic proportions in southwest China in late February, 1957 (1). During the next three months influenza outbreaks occurred widely throughout the Orient and first appeared in the United States in the middle of May. During the summer the disease spread slowly and sporadically within the United States, initially involved military establishments which received military personnel from the Orient, and later attacked various civilian groups collected in summer camps and conferences. From September through December, 1957, there was a rapid and diffuse spread of influenza, and epidemics were observed in most cities throughout the country (2).

In late August, 1957, it was predicted that influenza would probably be seen in epidemic proportions within New York City. At this time a prospective interdepartmental study was organized at The New York Hospital-Cornell Medical Center with the belief that the pending epidemic might present a unique opportunity to study influenza by a multidisciplined approach not often possible under epidemic conditions.

The present series of three papers is a report of these studies. The first paper details observations on an intrahospital epidemic arising within a single medical ward. Included in this initial paper of the report are the results of studies of the clinical syndrome and the clinical pathology of uncomplicated influenza, the results of different serologic methods for the diagnosis of influenza, and observations on the efficacy of prior influenza vaccination in protecting a small group of personnel heavily exposed to the infection. The second paper (3) analyzes the clinical course and the microbiological and pathologic characteristics of pneumonia arising in patients with Asian influenza A virus infection as witnessed at The New York Hospital from September, 1957, through January, 1958. The final paper (4) reports in detail the techniques used in the isolation of influenza virus and the laboratory characteristics of the strains of Asian influenza A virus recovered from patients during the present study.

METHOD OF STUDY

1. Persons included in the study group. Sixty-two persons were confined within, or working on, Ward H5 at the time of the epidemic. All were included in the present study. This group consisted of 29 male and female patients over 16 years of age admitted because of medical illnesses and a ward staff of 33 persons employed on various shifts. The personnel included four physicians, five medical students, seven registered nurses, five student nurses, nine nurses' aides, and three floor clerks.

2. Vaccination status of group. None of the 29 medical patients had received influenza vaccine. Twenty members of the H5 staff had received one or more injections of a monovalent vaccine containing 200 chicken-red cell-agglutinating (CCA) units per ml. of the Asian strain of influenza A virus. Sixteen persons had been given 1.0 ml. (200 CCA units) of vaccine subcutaneously 19 days before the appearance of influenza on the ward. Three persons had received intradermal injections of 0.1 ml. (20 CCA units) of vaccine 19 and 12 days prior to the influenza outbreak. One additional staff member received 0.2 ml. (40 CCA units) intradermally 2 days after the epidemic began.

* This work was supported in part by Traineeship Grant E-6 and Research Grants E-2162 and E-1078 from the National Institute of Allergy and Infectious Diseases, Public Health Service; The Research and Development Division, Office of the Surgeon General, Department of the Army under Contract Number DA-49-007-MD-703; and by grants from Chas. Pfizer & Co., Brooklyn, N. Y.; The Upjohn Co., Kalamazoo, Mich.; and Wyeth Laboratories, Philadelphia, Pa.

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3. Clinical data. Daily observations were made by the house staff and influenza study group on all patients who developed symptoms suggestive of influenza. A check sheet was used to facilitate uniform tabulation of daily symptoms and signs. Rectal temperatures and pulse rates were recorded every four hours. Similar studies were carried out on personnel who developed influenzal symptoms, but were of necessity less complete.

4. Routine laboratory tests. Total and differential leukocyte counts, hematocrit values, and erythrocyte sedimentation rates were determined every three days on subjects who developed influenza. Studies of liver and renal function, chest roentgenograms, and special serologic and bacteriologic studies were obtained in selected cases.

5. Viral and bacteriologic studies. Fifteen ml. of phosphate buffered saline solution (0.85 per cent NaCl) was gargled and collected for recovery of influenza virus. These washings were obtained from five patients within 24 to 96 hours after the onset of influenza symptoms. Techniques of virus isolation are described in a succeeding paper (4).

Nose and throat cultures were obtained from 53 of the 62 individuals on the third day of the epidemic whether or not symptoms or signs of influenza were apparent. Sputum specimens were obtained from all patients with productive cough. Sputum smears were stained by the Gram method and examined microscopically. Throat swabs and sputum specimens were streaked on blood agar plates and inoculated into beef heart infusion broth. Particular attention was given to the isolation and identification of coagulase positive staphylococci, pneumococci, *Hemophilus influenzae,* and beta hemolytic streptococci.

6. Serologic studies. Two or more specimens of serum were obtained from 55 of the 62 subjects of the study. Initial blood specimens were drawn on the fourth day following admission of the initial case of influenza. Subsequent blood specimens were obtained 5 to 21 days after this initial bleeding. Blood was allowed to clot at room temperature; the serum was separated and stored at −20° C. until studies were performed. Three serologic tests for influenza were performed on each set of sera, and paired sera were titrated simultaneously. Complement fixing (CF) antibodies were measured by a standard procedure (5), using as the test antigen an A/

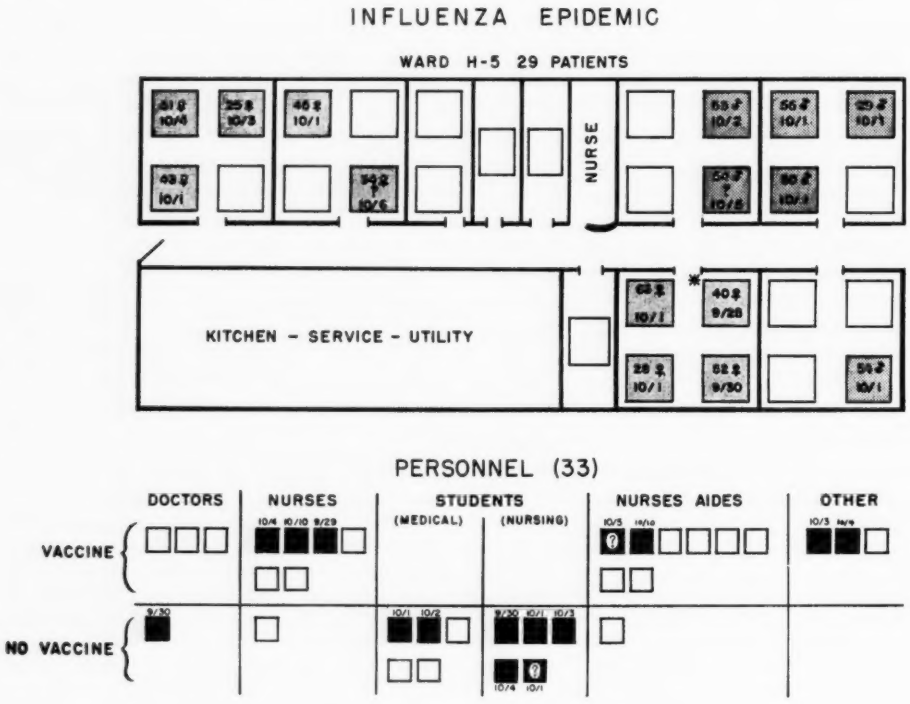

FIG. 1. TOPOGRAPHY OF WARD H5 AND MAKE-UP OF WARD PERSONNEL

Shaded blocks represent individual patients and personnel who developed influenza symptoms. The date of appearance of symptoms is indicated within or above the blocks. The location of the initial case is indicated by an asterisk.

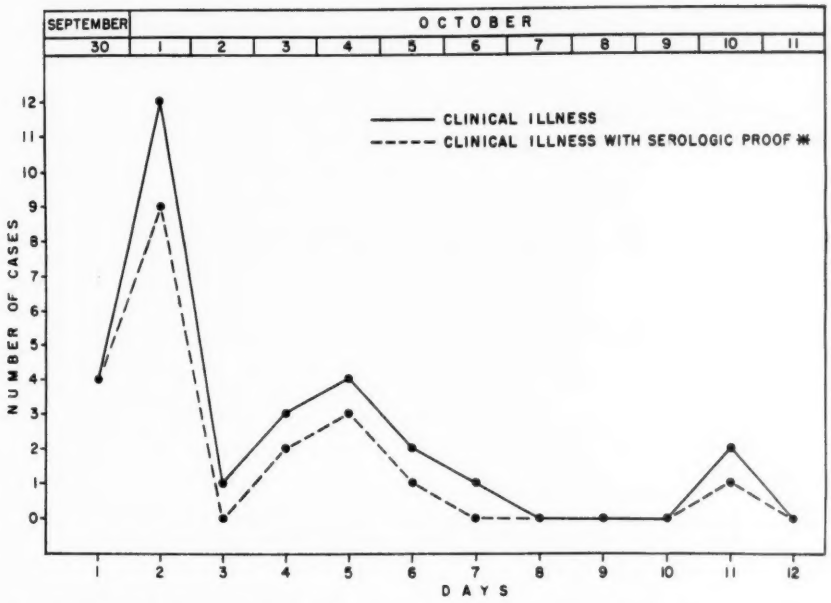

FIG. 2. DAILY APPEARANCE OF NEW CASES OF INFLUENZA ON WARD H5 BY DATE OF MONTH AND DAY OF EPIDEMIC

The term "serologic proof" is equated with a demonstrated fourfold or greater rise in hemagglutination-inhibiting or complement fixing antibodies.

Japan/305/57 strain which had been passaged only in eggs.[1]

Titration of hemagglutination-inhibiting (HI) antibody. Early in the present study HI antibody was measured with the egg-line strain of the Japan 305 (Asian strain) virus and trypsin inactivated sera. The relative insensitivity of this antigen to nonspecific serum inhibitor obviated the use of the more effective periodate method (see below) for the destruction of inhibitor. The egg-line virus was also relatively insensitive to inhibition by antibody, however, so that few antibody rises were demonstrated with this virus. Accordingly, most sera were re-titrated with an inhibitor and antibody-sensitive variant of the Japan 305 strain which had been passed through ferrets and mice (Japan 305 $E_4F_1M_3E_8$, obtained through the courtesy of Dr. Keith Jensen). In these latter titrations, preliminary destruction of inhibitor was effected with periodate as described below.

a) Inhibitor-resistant virus/trypsin inactivation of serum inhibitor. Equal parts of serum and two per cent trypsin in phosphate buffered saline (PBS) were heated together at 56° C. for 30 minutes. Serial twofold dilutions of 0.2 ml. volume of the inactivated serum were made in PBS. Two-tenths ml. of "egg-line" Asian influenza virus strain A/Japan/305/57 containing 16 to 32 hemagglutinating units was added to each tube. After 15 minutes' incubation at 22° C., 0.4 ml. of a 0.5 per cent suspension of human "O" red cells was added. The test was observed for inhibition of red cell agglutination after 40 minutes' incubation at 22° C.

b) Inhibitor-sensitive (mouse-ferret passaged) virus/periodate inactivation of serum inhibitor. Two-tenths ml. of serum was heated at 56° C. for 30 minutes. Four-tenths ml. of M/90 potassium periodate solution in distilled water was added to the serum after cooling, and the mixture refrigerated at 4° C. for 18 hours. Four-tenths ml. of 1 per cent glycerol in PBS was then added and the mixture maintained at room temperature for one hour to neutralize further periodate action. Serial dilutions and the remainder of the test procedure were the same as those used following treatment with trypsin. This method represents a modification of a procedure originally proposed by Burnet and Lind (6) and adapted by Jensen (7).

[1] This strain, obtained from Dr. John Y. Sugg, was initially isolated at the Army Medical School, Washington, D. C. It had been passaged once by the allantoic route in eggs prior to receipt. Three additional egg passages were carried out prior to its use in these studies.

RESULTS

Epidemiology

Ward H5 is composed of 29 beds arranged in one, two and four bed units as diagrammed in

TABLE I

Summary of information on individuals on Ward H5 developing influenzal symptoms

No.	Patient, Age, Sex	Status	Underlying disease*	Vaccine route,† Date	Date, onset of clinical symptoms	Duration of clinical symptoms	Severity of illness	Max. temp.	WBC/1,000 (range)	Specific antibody rise
						days		*°C.*		
1	R. S. 40 F	Patient	RHD, MS, MI, Auric. fibril.		9/28	8	3+	40.6	2.0–11.9	+
2	D. B. 24 M	MD	None		9/30	4	1+	39.0		++
3	L. F. 18 F	RN	None	S.C. 9/11	9/30	5	2+		13.9	++
4	S. F. 23 F	Stud. nurse	None		9/30	3	2+	38.3	5.1	++
5	B. N. 52 F	Patient	RHD, MS, MI, Heart failure		9/30	31	3+	39.8	6.3–11.4	++
6	J. B. 22 M	Med. stud.	None		10/1	1	1+			0
7	C. C. 63 M	Patient	Rheumatoid arthritis		10/1	6	1+	38.8	10.8	++
8	G. C. 54 M	Patient	Cirrhosis, Diabetes mellitus		10/1	2	1+	39.5	4.1– 6.7	++
9	M. D. 20 F	Stud. nurse	None		10/1	1	1+			0
10	S. D. 45 F	Patient	RHD, MI, Heart failure		10/1	7	2+	39.8	4.9– 7.3	++
11	J. G. 19 F	Stud. nurse	None		10/1	2	2+	39.6	3.7	++
12	V. G. 43 F	Patient	Hodgkin's dis., Rt. pleural effusion		10/1	7	2+	39.5	6.8– 9.2	0
13	E. L. 55 M	Patient	RHD, MS, MI, Auric. fibril., Ht. fail.		10/1	3	1+	39.3	6.9–11.5	++
14	R. K. 29 M	Patient	Rheumatic fever		10/1	6	1+	38.8	7.2–10.4	++
15	A. M. 28 F	Patient	Serum sickness		10/1	8	3+	40.0	5.2–14	++
16	K. N. 63 F	Patient	Myocardial infarction		10/1	2	1+	38.5	4.3– 5.3	++
17	G. V. 30 M	Patient	RHD, AI, Heart failure		10/1	8	3+	40.2	6.5–11.4	++
18	J. S. 23 M	Med. stud.	None		10/2	4	1+			?
19	E. C. 21 F	Stud. nurse	None		10/3	3	2+	38.3	9.6	+
20	M. R. 38 F	Ward clerk	None	S.C. 9/11	10/3	6	2+			++
21	M. T. 25 F	Patient	Anemia, Fever unknown origin		10/3	3	1+	38.5		?
22	M. E. 23 F	Stud. nurse	None		10/4	5	2+	38.6	6.2– 6.5	+
23	A. F. 24 F	RN	None	S.C. 9/11	10/4	4	2+			0
24	E. H. 51 F	Patient	Hypertension, Periph. vascular dis.		10/4	3	1+	39.3	9.2–10.3	++
25	L. S. 36 F	Ward clerk	None	S.C. 9/11	10/4	4	2+			++
26	I. C. 54 M	Patient	Hypertension, CVA, Pulm. insuff.		10/5	2	1+	39.6	8.1– 9.9	++
27	J. L. 25 F	Nurses' aide	None	S.C. 9/11	10/5	?	?			?
28	A. P. 34 F	Patient	Ulcerative colitis		10/6	4	1+	39.8		0
29	M. B. 30 F	RN	None	S.C. 9/11	10/10	4	1+			+
30	L. J. 21 F	Nurses' aide	None	I.D. 10/2	10/10	5	1+			?

* Abbreviations are as follows: RHD, rheumatic heart disease; MS, mitral stenosis; MI, mitral insufficiency; AI, aortic insufficiency; CVA, cerebrovascular accident.

† S.C., subcutaneous; I.D., intradermal.

Figure 1. Rooms open into a central ward corridor which communicates with the main hospital corridor by a single door. Patients leave the ward for special study procedures in other parts of the hospital, but many remain within the confines of the ward during their entire hospital stay. Personnel move freely between the ward, other parts of the hospital, and the outside community. At the time of the epidemic, all 29 beds were occupied.

On September 28, 1957, a 40 year old woman (R. S.) with known rheumatic heart disease was admitted to a four bed room on H5 with an undiagnosed acute febrile illness subsequently proved to be influenza complicated by bacterial pneumonia. Isolation procedures were not instituted. Within 24 hours symptoms of influenzal nature developed in the patient in the adjacent bed and in three of the ward staff. The subsequent spread of disease within the ward and the daily appearance of new cases are depicted in Figures 1 and 2.

During the next 24 hour period an additional 12 individuals developed an acute febrile illness believed to be influenza. Within seven days, 28 persons had developed symptoms consistent with a diagnosis of influenza. Two others developed symptoms typical of influenza on the eleventh day after the admission of the index case, but by this time influenza was prevalent within the community and the hospital, and these two cases may have arisen from an external source. Data on the 30 individuals who developed clinical influenza are recorded in Table 1.

Clinical manifestations of influenza

The clinical manifestations of influenza were strikingly uniform, and few differences were noted in the symptom complex manifested by patients or personnel. The incidence of predominating symptoms is given in Table II. In 29 of 30 individuals developing influenza, the onset was characterized by the relatively abrupt appearance of fever; temperature rose rapidly to maximum levels, commonly above 39° C., during the first 24 to 48 hours. Fever then subsided rapidly. Temperature elevations persisted from 48 to 96 hours in the absence of complications. Eight persons noted chilly sensations during the period of rising temperature. Cough of varying severity appeared within the first 24 hours in 25 individuals but was productive of sputum in only four. Three patients complained of moderate substernal pain associated with severe and protracted cough.

TABLE II

Incidence of symptoms in 30 individuals on Ward H5 manifesting influenza symptoms

Symptom	With significant antibody titer rise (21)	Without significant antibody titer rise (9)	Total (30)	
			Number	Percent
Fever	20	9	29	97
Cough	17	8	25	80
Headache	15	2	17	63
Nasal discharge	12	2	14	47
Sore throat	11	2	13	43
Gastrointestinal symptoms	7	3	10	33
Generalized aching	7	2	9	30
Chills	8	0	8	27
Chest pain	3	0	3	10
Epistaxis	2	0	2	7

During the first 24 hours of illness, 17 individuals developed frontal headache which appeared related in intensity to the height of the temperature rise. Generalized muscle aches were noted more frequently by personnel than by bed patients and were not a prominent feature of this illness. Thirteen persons complained of a scratchy sore throat, but on examination only minimal inflammation of the pharyngeal mucosa was noted in 10. Mild nasal discharge appeared in 14 individuals; the nasal mucosa was visibly inflamed in six. Two patients with rhinitis had single episodes of epistaxis.

Ten persons with influenza had complaints referable to the gastrointestinal system; of these persons, seven were previously hospitalized bed patients and three were members of ward personnel. Although two previously healthy persons suffered nausea and vomiting, the occurrence of diarrhea or abdominal pain was restricted in all but one case to bed patients with antecedent disease. Similarly, the incidence of nausea and vomiting was two to three times higher among bed patients than in personnel. Of the seven of 15 bed patients with gastrointestinal symptoms, four had rheumatic heart disease (three with cardiac failure), and others had diagnoses of serum sickness, Hodgkins disease, and rheumatic fever. In addition, all but one patient were receiving medication of possible importance in the genesis of gastrointestinal symptomatology. These drugs included

oral penicillin, sulfonamides, erythromycin, codeine sulfate, and tetracycline, as well as digitalis in those patients with heart failure.

The occurrence of mild gastrointestinal symptoms in only three of 15 ward personnel is more in keeping with the rarity of abdominal complaints observed in previous clinical studies of influenza (8, 9).

Prostration, depression, and malaise which appeared out of proportion to the severity of the illness were more common in patients than in personnel. Of interest was the relatively greater severity of illness in those patients with underlying rheumatic heart disease. As shown in Table I, of the 30 persons manifesting symptoms of influenza, five had rheumatic heart disease, 10 had been hospitalized for other disease processes, and 15 were previously healthy personnel. Three of the five patients with rheumatic heart disease had severe influenza symptoms and developed pulmonary complications. Among the other 25 individuals studied, 10 with nonrheumatic illness and 15 without underlying disease, only one case of severe influenza occurred. This patient did not develop manifest pulmonary disease. Although the personnel group was younger, the patients with chronic nonrheumatic illnesses were of an age comparable to those with rheumatic heart disease.

Physical findings of lower respiratory tract involvement were noted in five individuals. Two patients developed fine, moist bilateral basilar rales without roentgenographic evidence of pulmonary infiltrates. These signs disappeared spontaneously within three days. Two bed patients with severe rheumatic heart disease developed clinical roentgenographic evidence of pneumonia during the course of influenza. A third patient with rheumatic heart disease developed persistent pleural pain with an audible friction rub bilaterally. These three patients (R. S., G. V. and B. N.) are discussed in the succeeding paper (3).

Laboratory studies

Leukocyte counts. As noted in Figure 3, no consistent changes in leukocyte counts were noted in patients with influenza. Total leukocyte counts

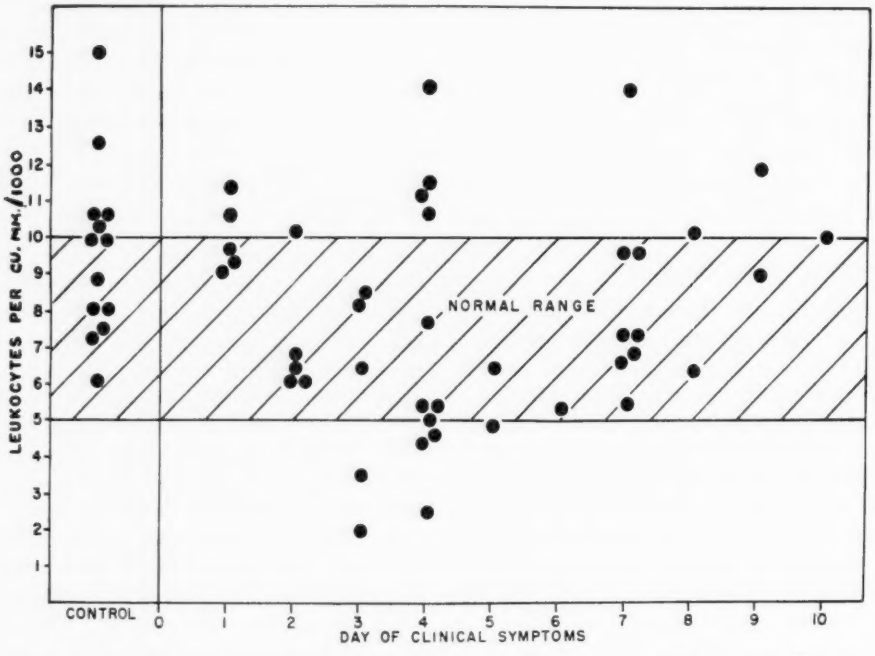

FIG. 3. TOTAL LEUKOCYTE COUNTS IN INDIVIDUALS WITH INFLUENZA IN RELATION TO ONSET OF SYMPTOMS

Values of leukocyte counts obtained on this group prior to influenza are also given.

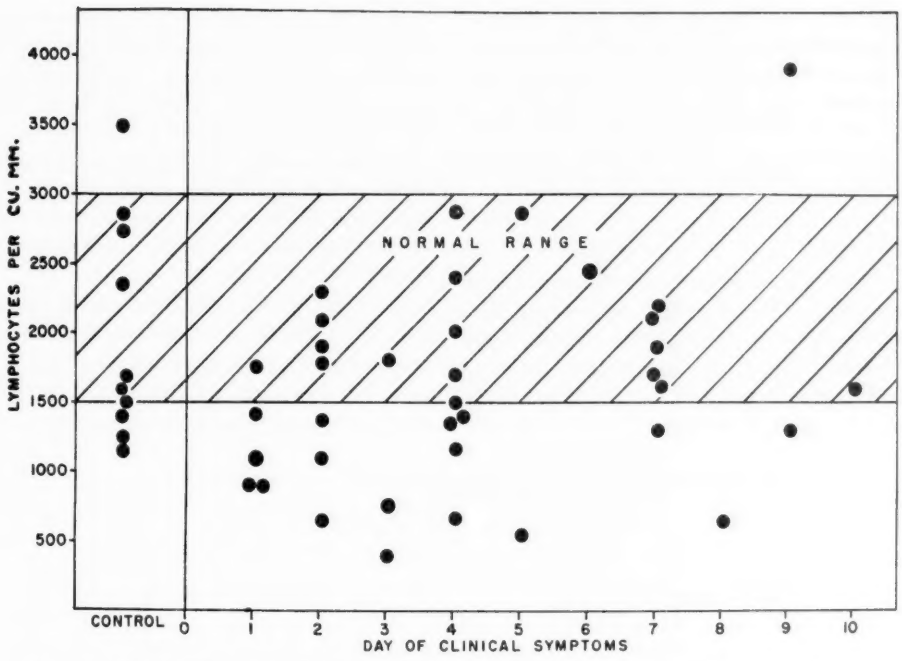

FIG. 4. TOTAL LYMPHOCYTE COUNTS IN INDIVIDUALS WITH INFLUENZA IN RELATION TO ONSET OF SYMPTOMS

Values of lymphocyte counts obtained on this group prior to influenza are also given.

obtained during the disease period varied from 2,000 to 14,000 cells per cu. mm. Nine of 18 patients had leukocyte counts which remained consistently within the normal range. Four persons had transient leukopenia which occurred on the third and fourth day of illness. A leukocytosis of 14,000 cells per cu. mm. was encountered in two patients. The three patients who developed pulmonary bacterial complications could not be differentiated on the basis of leukocytic response. Lymphopenia was common during the first four days of influenza in the face of normal total leukocyte values. Lymphocyte counts are graphed in Figure 4. Striking lymphopenia was noted in four individuals. The single instance of sustained lymphopenia was associated with a normal total leukocyte count.

Erythrocyte sedimentation rates. Erythrocyte sedimentation rates were commonly elevated during the course of influenza, but a significant number of hospitalized patients had increased sedimentation rates prior to the onset of influenza, rendering uncertain the significance of this observation.

Bacteriologic studies

Nose and throat cultures were obtained from 53 persons during the course of the epidemic. These cultures did not demonstrate significant differences in the recovery of potential pathogens from individuals with influenza and those without influenzal symptoms. Cultural data are presented in Table III. Pneumococci and *Hemophilus influenzae*

TABLE III

Bacteriologic findings in nose and throat cultures obtained from 53 individuals

Organism	With influenzal symptoms (24)	Without influenzal symptoms (29)
Coagulase positive staphylococcus aureus	8	13
Pneumococcus	4	1
Hemophilus influenzae	4	0
Group A streptococcus	0	0

were isolated from four persons with influenza. Pneumococcal pneumonia was present in one of these patients. Coagulase positive staphylococci were isolated from eight persons with influenza and 13 without evidence of disease. Beta hemolytic streptococci were not recovered from any patient.

Virologic studies

Virus isolation. Because of the explosive nature of the outbreak, specimens for virus isolation were not obtained until 96 to 120 hours after the onset of the epidemic, and only five attempts at virus isolation were performed on selected recent cases. The Asian strain of influenza A virus was recovered from a throat washing obtained 96 hours after the onset of influenza in one patient. Throat washings obtained from the remaining four patients, 24 to 96 hours after the onset of symptoms, did not yield influenza virus.

Serologic studies. Fifty-five paired serum specimens were available for serologic study. A fourfold rise in antibody titer between acute and convalescent serums was considered evidence of influenza virus infection.

Fifty-four pairs of sera were tested for hemagglutination-inhibiting antibodies using trypsinized sera and egg-line, inhibitor-resistant virus. Initial titers were low, ranging from less than 1 : 8 to 1 : 32, and in none of the convalescent sera did the anti-

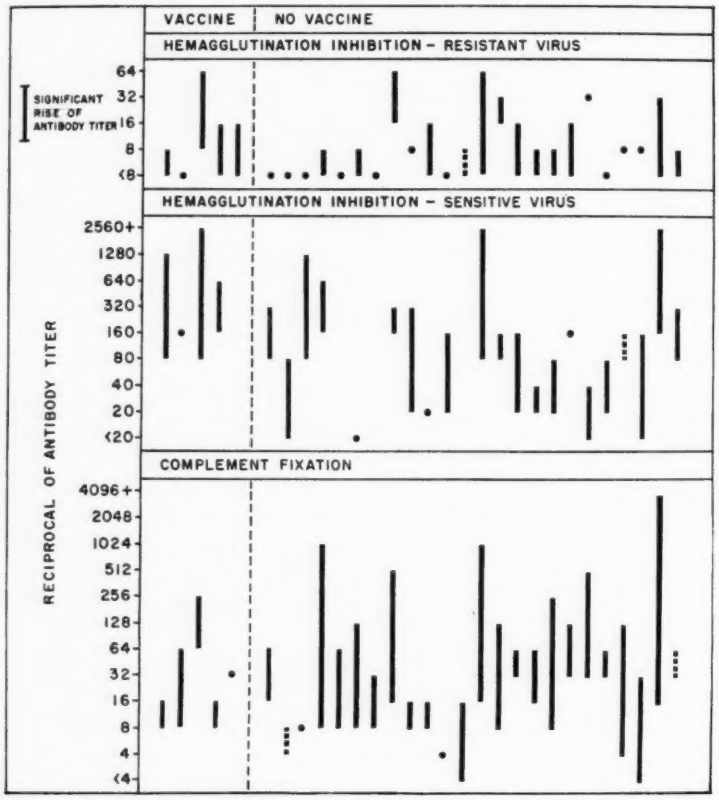

FIG. 5. COMPARISON OF CHANGES IN ANTIBODY TITER BY THREE SEROLOGIC METHODS IN INDIVIDUALS IN WHOM ANTIBODY TITER RISE WAS DEMONSTRATED BY ANY ONE OF THESE METHODS

A solid line indicates a rise in titer, a dotted line a fall, and a single dot denotes that no change in titer occurred.

TABLE IV

Serologic findings on individuals manifesting clinical influenza and/or significant antibody rise

No.	Case	Date, onset of symptoms	Vaccination route, Date	Date of sera		Reciprocal of antibody titers						Significant antibody rise		
				Early	Convalescent	HI-inhibit. resis. virus		HI-inhibit. sens. virus		Comp. fix.		HI-resis. virus	HI-sens. virus	Comp. fix.
1	R. S.	9/28		10/3	10/16	<8	32	160	2,560+	16	4,096+	+	+	+
2	D. B.	9/30		10/3	10/16	<8	<8	80	320	16	64	0	+	+
3	L. F.	9/30	S.C. 9/11	10/4	10/16	<8	16			32	32	+		0
4	S. F.	9/30		10/4	10/16	<8	8	<20	<20	8	128	0	0	+
5	B. N.	9/30		10/3	10/16	<8	64	80	2,560+	16	1,024	+	+	+
6	J. B.	10/1		10/3	10/16	<8	<8	80	80	16	16	0	0	0
7	C. C.	10/1		10/3	10/8	<8	<8	20	160	4	4	0	+	0
8	G. C.	10/1		10/3	10/16	8	<8			<4	16	0		+
9	M. D.	10/1		10/3	10/16	8	<8	<20	<20	8	8	0	0	0
10	S. D.	10/1		10/3	10/16	<8	16	20	20	8	16	+	0	0
11	J. G.	10/1		10/4	10/16	<8	<8			8	32	0		+
12	V. G.	10/1		10/3	10/16	16	<8			<4	<4	0		0
13	E. L.	10/1		10/3	10/16	16	64	160	320	16	512	+	0	+
14	R. K.	10/1		10/3	10/24	<8	<8	20	80	32	64	0	+	0
15	A. M.	10/1		10/3	10/8	16	32	80	160	8	128	0	0	+
16	K. N.	10/1		10/3	10/24	<8	16	160	160	32	128	+	0	+
17	G. V.	10/1		10/3	10/16	32	32	<20	40	32	512	0	+	+
18	J. S.	10/2												
19	E. C.	10/3		10/3	10/16	<8	<8	160	640	8	1,024	0	+	+
20	M. R.	10/3	S.C. 9/11	10/3	10/16	<8	<8	160	160	8	64	0	0	+
21	M. T.	10/3												
22	M. E.	10/4		10/3	10/16	<8	<8			8	64	0		+
23	A. F.	10/4	S.C. 9/11	10/3	10/16	<8	<8	80	80	64	64	0	0	0
24	E. H.	10/4		10/3	10/16	8	8	20	320	8	16	0	+	0
25	L. S.	10/4	S.C. 9/11	10/3	10/24	8	64	80	2,560+	64	256	+	+	+
26	I. C.	10/5		10/3	10/24	<8	8	20	80	8	256	0	+	+
27	J. L.	10/5	S.C. 9/11											
28	A. P.	10/6		10/3	10/16	16	8	320	320	16	16	0	0	0
29	M. B.	10/10	S.C. 9/11	10/3	10/17	<8	16	160	640	8	16	+	+	0
30	L. J.	10/10	I.D. 10/2	10/2	11/1			40	160				+*	
31	G. B.		I.D. 9/11	10/3	10/16	<8	8	80	1,280	8	16	0	+	0
32	D. D.			10/3	10/16	<8	<8	80	1,280	8	8	0	+	0
33	Z. H.			10/3	10/16	<8	16	20	160	32	64	+	+	0
34	R. K.			10/3	10/16	<8	<8	<20	80	8	4	0	+	0
35	F. L.			10/3	10/16	<8	8	20	40	16	64	0	0	+
36	V. N.			10/3	10/16	8	8	160	80	4	128	0	0	+
37	A. S.			10/3	10/16	8	8	<20	160	<4	32	0	+	+
38	K. W.			10/3	10/17	<8	8	80	320	64	32	0	+	0

* This antibody rise was assumed to be due to vaccination.

body titer rise above 1:64. A significant rise in antibody titer was demonstrated in only nine pairs of sera by this method. All titers in this paper are expressed as final dilutions of serum.

Because of the apparent insensitivity of the egg-line virus in demonstrating antibody response, tests were repeated using potassium periodate for inhibitor destruction and a ferret-mouse passaged virus sensitive to both inhibitor and antibody. Forty-eight tests on paired sera were performed by this method which proved a more sensitive technique for the demonstration of influenza infection. Significant titer rises were demonstrated in 18 cases. The high initial titers of apparent antibody in some sera suggest that all inhibitor was not necessarily destroyed with periodate. However, the increased number of titer increases demonstrated with this method affirm its usefulness. Titration of antibody with a virus strain isolated from the epidemic (Case S. D.) did not demonstrate additional serologic responses in cases negative with the ferret-mouse line (inhibitor-sensitive) virus.

Fifty-two pairs of sera were tested for the presence of complement fixing antibody with viral antigen of the egg-line Japan 305 influenza virus. Significant increases in titer were evident with 18 pairs of sera.

Increases in both hemagglutination-inhibiting and complement fixing antibodies were noted as early as seven days after the onset of influenza symptoms, in accord with earlier observations on the appearance of neutralizing antibodies in influenza (10).

As noted in Figure 5, patients manifesting significant antibody response by one method did not necessarily demonstrate antibody rise by other serologic methods. Utilizing all three serologic tests for diagnosis, proof of influenza infection was obtained in 21 of 30 individuals developing clinical disease. In addition, eight persons who showed no clinical manifestations of disease had significant rises in antibody titers, indicating that inapparent influenza infection occurred during the epidemic period. A full summary of serologic data obtained on all individuals manifesting either the clinical syndrome or serologic evidence of recent influenza infection is presented in Table IV.

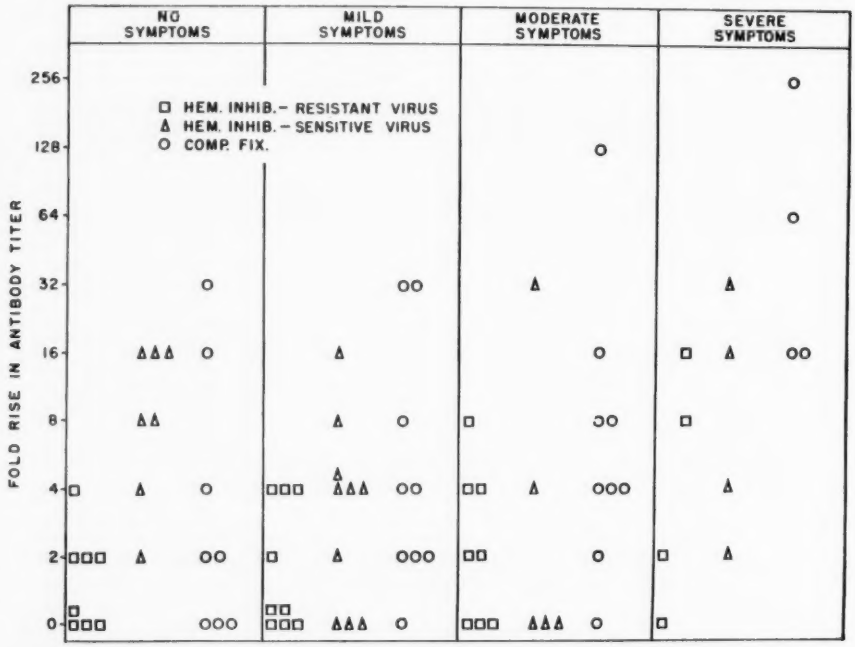

FIG. 6. CORRELATION OF FOLD RISE IN ANTIBODY TITER AND THE SEVERITY OF CLINICAL ILLNESS

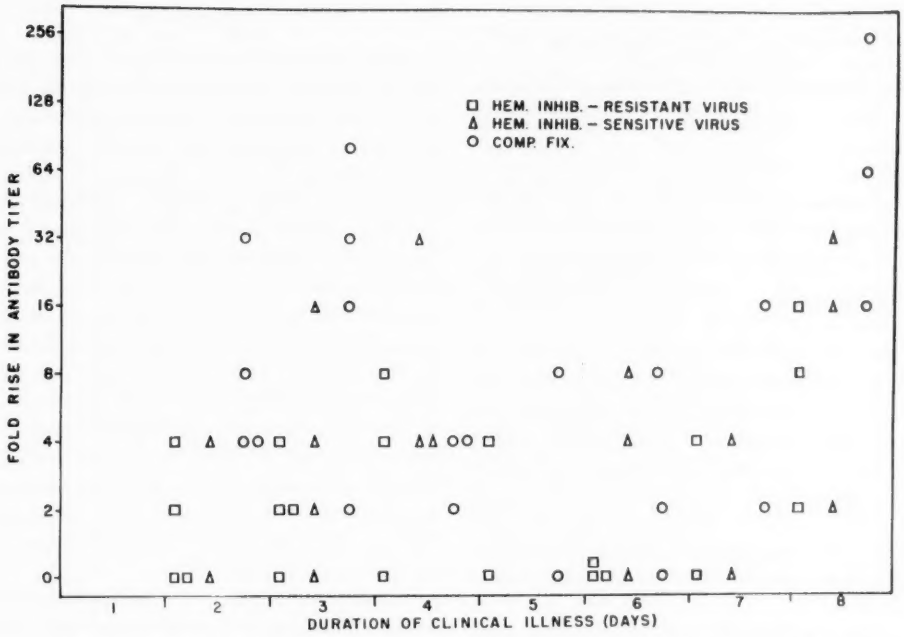

FIG. 7. CORRELATION OF FOLD RISE IN ANTIBODY TITER AND THE TOTAL DURATION OF THE CLINICAL ILLNESS

The relation of the degree of antibody rise to the severity and duration of influenza symptoms

The relation of antibody response to the severity and duration of influenzal symptoms was examined. The relevant data are presented in Figures 6 and 7. It is evident that the spread of antibody response is broad and the data scattered, particularly when antibody response and duration of illness are correlated. However, if attention is directed to individual CF antibody responses of fourfold or more, a definite trend is noted in those cases with proved infection. This trend of *increasing incidence of significant antibody response with increasing severity of infection* could merely indicate *more accurate clinical diagnosis* in the more severely ill. That this interpretation is partially correct is shown by comparison of the geometric means of the antibody responses of fourfold or more in the various categories of illness. It is found that these average fold increases are 12.1 in patients with "no symptoms," 10.6 in patients with "mild symptoms," and 9.2 in patients with "moderate symptoms." In the few patients with "severe symptoms," however, significantly greater response, averaging 45-fold, was found. Thus the antibody response of seriously ill patients clearly exceeded that of patients with milder symptoms even when comparison was restricted to those with serologically proved influenza.

Incidence of influenza in vaccinated and nonvaccinated individuals

As previously noted, 20 staff members had received influenza vaccine prior to the appearance of influenza on H5. In 19, sufficient time (19 days) had elapsed for the development of measurable antibody (11).

Pre-epidemic antibody levels in vaccinated individuals were comparable to those demonstrated in nonvaccinated individuals when determined by complement fixation or hemagglutination-inhibiting antibody studies using inhibitor-resistant virus. Higher levels of pre-epidemic antibody were noted in the majority of vaccinated individuals when hemagglutination-inhibiting antibody was determined with the inhibitor-sensitive virus antigen

TABLE V

Incidence of influenza in vaccinated and nonvaccinated individuals

Group	No. developing clinical influenza / No. at risk	No. with significant antibody titer rise / No. tested
Vaccinated	7/20 (35%)	5/16 (31%)
Nonvaccinated	23/42 (55%)	24/37 (65%)

(Figure 5, Table IV). Nevertheless, there was considerable overlap with the titers obtained in nonvaccinated individuals.

Despite the equivocal nature of antibody increase in vaccinated individuals, suggestive evidence of vaccine protection was obtained. As noted in Table V, seven of 20, or 35 per cent of the vaccinated personnel developed clinical influenza compared with 23 of 42, or 55 per cent of the total nonvaccinated group. If only individuals developing significant antibody response were included in the analysis, 5 of 16, or 31 per cent of the vaccinated and 24 of 37, or 65 per cent of the total nonvaccinated group had documented influenza infection. If the 33 ward personnel were considered separately, 8 of 13 nonvaccinated staff members manifested clinical influenza in contrast to 7 of 20 individuals receiving influenza vaccination. Thus although the groups were small, the results suggest protection by immunization in a situation of concentrated exposure.

Miscellaneous laboratory studies

Laboratory studies did not reveal evidence of significant changes in liver or renal function. None of the patients tested developed serum cold agglutinins during influenza.

Studies on certain experimental bacterial infections suggest that profound changes in serum amino acids can occur during the course of generalized infection (12). The prostration associated with influenza led us to determine whether gross changes in serum proteins or serum amino acid levels accompanied the acute illness. Sera obtained from eight acutely ill patients were paired with sera from the same patients obtained following recovery and subjected to protein electrophoresis and paper chromatography. No differences could be detected in the patterns obtained during acute and recovery phases.

DISCUSSION

Explosive onset and swift spread of the infection in crowded populations are cardinal features of epidemic influenza. These characteristics suggested the etiology of the present epidemic and retrospectively led to the diagnosis of the patient admitted with fever of unknown origin who represented the primary case. The high attack rate of symptomatic infection produced by the pandemic virus of 1957 which involved 30 of 62 persons in this institutionalized group also resembled previous epidemics caused by other strains of influenza A virus (10). So, too, the incidence of clinically inapparent infection detected by specific antibody response (28 per cent) corresponded exactly with past experience reported by Horsfall, Hahn and Rickard (10) and closely with the 32 per cent incidence of inapparent influenza A noted by Kilbourne, Anderson and Horsfall in a mixed A and B epidemic in children (13).

The present outbreak occurred in a heterogeneous group containing both healthy and sick adults of widely differing ages. It can be assumed with reasonable certainty that infection was produced by a single strain of virus. It thus seems of particular interest to note the varying disease syndromes observed in different individuals. In young and previously healthy adults influenza virus infection caused the expected acute and prostrating but brief and benign illness typical of past epidemics of influenza A (8–10). In patients hospitalized with debilitating disease states, severe and unusual symptomatology and illness occurred. In these individuals, already ill, an undue incidence of gastrointestinal symptomatology was associated with the more usual symptoms of influenza virus infection. Furthermore, five hospitalized patients developed evidence of lower respiratory tract involvement. Although bacteriologic studies failed to reveal changes in the bacterial flora of the nasopharynx in the majority of persons developing influenza, three of these five patients developed definite bacterial pneumonia. Each of these three patients had underlying chronic rheumatic heart disease. The relationship between serious pulmonary complications of influenza and underlying illness is discussed in detail in the succeeding paper (3).

It thus appeared that the variations in severity

of influenza observed among members of this semi-closed community were probably related to differences in host management of infection rather than to variations in viral virulence during this small explosive outbreak.

While total leukopenia was uncommon, an absolute lymphocytopenia was noted to occur during the first four days of illness in many patients. This finding, reported in experimental influenza in human volunteers (14) and in murine infection (15, 16), deserves further investigation as a difference in the host reaction to viral and bacterial infections of possible diagnostic value. An extensive series of other laboratory studies did not reveal consistent or diagnostically useful abnormalities.

The present study supports the finding of Jensen and Hogan that multiple serologic methods are necessary for the maximum diagnosis of cases of influenza (17). Despite the use of three methods, significant antibody response was not demonstrated in nine patients who developed clinical illness indistinguishable from the serologically confirmed influenza of others. Although it is possible that these individuals contracted a respiratory infection of other etiology, the clinical syndrome and the setting in which it arose make it more likely that the serologic techniques used were inadequate to detect the presence of influenza in 30 per cent of those who contracted the disease.

Patients who were severely ill showed a greater antibody response than patients with mild or moderate symptomatology. This finding has not been previously documented in human influenza. Greater antigenic stimulation produced by the protracted presence of the virus or the attainment of greater viral (antigenic) mass during the course of the infection appear the most likely explanations of this phenomenon.

Evidence obtained in the present outbreak suggests that prior vaccination with small amounts of specific monovalent influenza vaccine may exert a protective effect in a group subjected to concentrated exposure. Serologically documented influenza infection occurred in 31 per cent of vaccinated individuals, as contrasted with 65 per cent of persons who had not received the vaccine. Although the difference in attack rate is not marked, this decrease in incidence of infection correlates well with previous studies on vaccine efficacy in small closed populations (18).

Thus in the present experience, the epidemiology and clinical nature of a small epidemic of influenza A within the 1957–58 pandemic resembled previous outbreaks occurring in interpandemic years. Detailed study of this epidemic has emphasized the influence of the underlying status of the human host on the severity of the disease, the frequency of lymphopenia early in the course of influenza, and a correlation between the severity of the illness and the magnitude of specific antibody response.

SUMMARY

1. An epidemic of influenza A on a hospital ward during the pandemic of 1957–58 caused illness in 30 persons of a total population of 62. Serologic evidence of influenza infection was obtained in eight persons who did not manifest symptoms of influenza.

2. The disease closely resembled previous descriptions of typical influenza, except for the occurrence of a high rate of gastrointestinal symptoms in patients with underlying disease. Three patients with rheumatic heart disease developed pulmonary complications of influenza.

3. Except for inconstantly observed lymphocytopenia during the first four days of illness, consistent abnormalities were not found with routine and special laboratory tests.

4. The incidence of serologically confirmed influenza was 34 per cent less in vaccinated than in nonvaccinated subjects.

5. Determination of both hemagglutination-inhibiting and complement fixing antibodies increased the number of cases diagnosed.

6. Although the severity and duration of illness were not correlated with antibody response as a straight line function, the mean magnitude of complement fixing antibody increase in seriously ill patients was almost fourfold greater than in other patients with significant serologic response to infection.

REFERENCES

1. Dunn, F. L. Pandemic influenza in 1957. Review of international spread of new Asian strain. J. Amer. med. Ass. 1958, **166**, 1140.
2. United States Communicable Disease Center. Influenza surveillance reports, Nos. 1 to 20. July 9 to Oct. 8, 1957.

3. Louria, D. B., Blumenfeld, H. L., Ellis, J. T., Kilbourne, E. D., and Rogers, D. E. Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. J. clin. Invest. 1959, **38**, 213.
4. Kilbourne, E. D. Studies on influenza in the pandemic of 1957–1958. III. Isolation of influenza A (Asian strain) viruses from influenza patients with pulmonary complications. Details of virus isolation and characterization of isolates, with quantitative comparison of isolation methods. J. clin. Invest. 1959, **38**, 266.
5. American Public Health Association. Diagnostic Procedures for Viral and Rickettsial Diseases, 2nd ed. New York, American Public Health Association, 1956, p. 258.
6. Burnet, F. M., and Lind, P. E. Recombination of influenza viruses in the de-embryonated egg. I. The use of periodate-treated sera for *in vitro* characterization of influenza virus strains. Aust. J. exp. Biol. med. Sci. 1954, **32**, 145.
7. Jensen, K. E., Dunn, F. L., and Robinson, R. Q. Influenza, 1957. A variant and the pandemic. Progr. med. Virol. 1958, **I**, 165.
8. Stuart-Harris, C. H., Andrewes, C. H., and Smith, W. A study of epidemic influenza: With special reference to the 1936–37 epidemic. Medical Research Council, Special Report Series, No. 228. London, His Majesty's Stat. Off., 1938.
9. Kilbourne, E. D., and Loge, J. P. Influenza A prime: Clinical study of an epidemic caused by a new strain of virus. Ann. intern. Med. 1950, **33**, 371.
10. Horsfall, F. L., Jr., Hahn, R. G., and Rickard, E. R. Four recent influenza epidemics: An experimental study. J. clin. Invest. 1940, **19**, 379.
11. McCarroll, J. R., and Kilbourne, E. D. Immunization with Asian strain influenza vaccine. Equivalence of the subcutaneous and intradermal routes. New Engl. J. Med. 1958, **259**, 618.
12. Woodward, J. M., Sbarra, A. J., and Holtman, D. F. The host-parasite relationship in tularemia. I. A study of the influence of Bacterium tularense on the amino acid metabolism of white rats. J. Bact. 1954, **67**, 58.
13. Kilbourne, E. D., Anderson, H. C., and Horsfall, F. L., Jr. Concurrent infection with influenza A and B viruses in a single epidemic of influenza. J. Immunol. 1951, **67**, 547.
14. Smorodintseff, A. A., Tushinsky, M. D., Drobyshevskaya, A. I., and Korovin, A. A. Investigations on volunteers infected with the influenza virus. Amer. J. med. Sci. 1937, **194**, 159.
15. Cary, G. R., Sorrell, W. B., and Kilbourne, E. D. The leukopenia of virus infection—Relation to the concentration of virus and the viral lesion (abstract). Amer. J. Med. 1955, **19**, 137.
16. Merkatz, I., Attia, A., and Kilbourne, E. D. Unpublished data.
17. Jensen, K. E., and Hogan, R. B. Laboratory diagnosis of Asian influenza. Publ. Hlth Rep. (Wash.) 1958, **73**, 140.
18. Hirst, G. K., Rickard, E. R., and Friedewald, W. F. Studies in human immunization against influenza. J. exp. Med. 1944, **80**, 265.

STUDIES ON INFLUENZA IN THE PANDEMIC OF 1957–1958. II. PULMONARY COMPLICATIONS OF INFLUENZA *†

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Influenza presents a paradox. To the clinician practicing medicine in 1918, influenza was a fearsome disease attended by frequent and often fatal pulmonary complications. To the student of interpandemic influenza in the last quarter century, the disease is an acute, temporarily incapacitating infection of the upper respiratory tract which is benign except on the rare occasion when bacterial pneumonia supervenes. This contrast in the manifestations of influenza has led to speculation that the disease of 1918 was either a different disease entity or caused by an agent of greater virulence than influenza viruses now encountered.

The microbiologic and pathologic evidence accumulated during the 1918–19 pandemic established the probable bacterial etiology of most influenza fatalities. There were, however, many clinicians who insisted that certain patients died of fulminating illness unassociated with known bacterial pathogens. This impression received support in the studies of Goodpasture who presented pathologic evidence of a diffuse hemorrhagic pneumonia in two patients which was unassociated with the demonstrable presence of bacteria (2).

The pandemic of 1918–19 antedated the first recognition of the influenza A virus (Smith, Andrewes and Laidlaw, 1933 [3]). Thus, knowledge of influenza derived from modern virologic studies of the epidemic (interpandemic) disease must be applied with caution to the 1918–19 pandemic. In the new pandemic in 1957, certain old questions remained unanswered:

1. What is the etiologic agent of pandemic influenza?

2. Is the pandemic disease more severe than the interpandemic form or only more widespread?

3. Is bacterial pneumonia the major cause of fatalities in pandemic influenza; if so, may fatalities be prevented by modern antimicrobials?

4. May influenza virus induce fatal disease in the absence of bacterial pathogens?

The first question has been unequivocally answered. A virus biologically indistinguishable from viruses previously isolated from cases of epidemic influenza may cause pandemic disease. The etiological agent of the 1957–58 pandemic, the so-called Asian strain of influenza virus, differs antigenically from previously isolated viruses, but is nonetheless a strain of influenza A virus. Furthermore, recent serologic studies suggest antigenic similarity of the virus of the 1957 pandemic to the agent of the pandemic of 1889 (4).

The definitive answer to the second question must await analysis of data from the present pandemic, but preliminary evidence suggests that pandemic influenza does not differ clinically from the epidemic disease (5, 6).

Partial answers to the final questions are subjects of the present paper. This investigation of patients with pulmonary complications of influenza was organized in the fall of 1957 as a prospective study of disease in the new pandemic. The results represent a collaborative integration of laboratory and bedside investigations.

During the 1957–58 influenza pandemic more than 50 patients with influenza and evidence of lower respiratory tract involvement were admitted

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† A preliminary report of a part of this investigation has been presented before the Association of American Physicians and has been published in the Transactions of that society (1).

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to The New York Hospital. Extensive clinical and laboratory observations were made on 33 individuals in this group in whom influenza virus infection could be clearly documented. As these studies progressed it became apparent that four pulmonary syndromes could be differentiated on the basis of history, physical examination, the appearance of chest X-rays, bacteriologic and virologic studies and the subsequent course of the illness. These syndromes have been categorized as follows:

1. Influenza virus infection with physical signs of lower respiratory tract involvement without infiltrates detectable by roentgenography.

2. Influenza virus infection followed by secondary bacterial pneumonia.

3. An acute, rapidly progressive pneumonia apparently produced by influenza virus infection alone.

4. Concomitant viral and bacterial pneumonia.

The present paper describes and defines these clinical entities. Included in this report are the results of laboratory and physiologic studies, the course of illness, the therapy employed and the microbiologic and histologic studies of tissues obtained post mortem.

MATERIALS AND METHODS

All 33 patients were hospitalized at The New York Hospital. Thirty patients were studied on the medical wards. Three pregnant women were studied on the obstetrical service.

1. Clinical studies. Each patient was initially examined by the house staff. When the clinical history was compatible with influenza, the patient was re-examined and evaluated by a member of the influenza study group. During the acute phase of illness, each patient was examined at least once daily by members of the study group who kept detailed records on symptoms, physical findings and the course of the illness.

2. Routine laboratory studies. Certain routine studies were performed serially on all patients. These included hematocrit determinations, total and differential leukocyte counts, erythrocyte sedimentation rates, urinalyses, chest roentgenograms, blood urea nitrogen, venous carbon dioxide combining power and serum sodium, serum potassium and serum chloride determination.

3. Virologic studies. The procedures used for the isolation of influenza virus are presented in detail in the succeeding paper (7). Throat washings were obtained for influenza virus isolation on the majority of patients. Attempts were also made to recover influenza virus from specimens of peripheral blood, urine and spinal fluid in selected cases. In patients dying during the course of their disease, specimens of lung tissue removed at autopsy were frozen and stored at $-68°$ C. until virus studies could be performed.

Acute serum specimens were drawn at the time of admission on all patients. Subsequent convalescent sera were obtained on one or more occasions 7 to 35 days later on patients who survived their illness. Sera were tested for the presence of hemagglutination-inhibiting and/or complement fixing antibody to the Asian strain of influenza A virus by techniques detailed in the preceding paper (5).

The following criteria were arbitrarily established as definitive evidence of recent infection with the Asian strain of influenza A virus:

1. A fourfold or greater rise in complement fixing antibody between acute and convalescent sera using the Japan 305 strain of influenza A virus as the test (viral) antigen.

2. An initial complement fixing antibody titer of 1:128 or greater against the Japan 305 strain in patients seen on or after the seventh day of influenza symptoms. These titers were felt to represent clear evidence of influenza infection after study of a series of 55 normal individuals revealed none with baseline serum titers of complement fixing antibody above 1:64.

4. Bacteriologic studies. Specimens of sputum, blood, and swabs from the nasopharynx and throat were cultured from all patients. In many instances, duplicate cultures were performed within the diagnostic bacteriology laboratory of The New York Hospital and the laboratories of the Division of Infectious Disease.

Sputum specimens were plated on blood and chocolate agar plates and inoculated into liver dextrose and thioglycollate broth. Particular attention was directed to isolation of coagulase positive staphylococci, pneumococci, *Hemophilus influenzae* and beta hemolytic streptococci. Microorganisms were identified by appropriate subculture, biochemical reactions, staining characteristics and serologic studies.

In many instances, 1 ml. of emulsified sputum was also inoculated intraperitoneally into white mice. When animals died, the peritoneal cavity was washed with 1 ml. of sterile saline and the peritoneal exudate examined by Gram stain and inoculated upon blood agar plates and into appropriate liquid media. Animals which survived sputum inoculation were killed several days later and the peritoneal washings subjected to the same procedures.

In fatal illnesses, postmortem cultures of lung and heart's blood were obtained from all but one patient. Specimens of lung tissue were plated directly on blood agar plates and inoculated into beef heart infusion broth, liver dextrose broth, and beef heart infusion broth containing five per cent defibrinated rabbits' blood. Specimens of lung from five patients were ground under sterile conditions, and quantitative pour plates were made to ascertain the concentrations of bacteria per milliliter of lung tissue.

All staphylococcal isolates were studied for pigment

production, hemolysis, mannitol fermentation and coagulase production. The majority of strains were subjected to bacteriophage typing using the technique of Blair and Carr (8).

Antibiotic sensitivity studies were carried out on all strains of staphylococci. Initial tests were performed utilizing the surface disc method. The discs used contained 10 μg. of tetracycline, chloramphenicol, streptomycin and erythromycin, 1.5 units of penicillin, and 5 μg. of novobiocin. In addition, standard serial tube dilution sensitivity studies were carried out on the majority of staphylococcal isolates. Beef heart infusion broth containing five per cent defibrinated rabbit blood was used as the medium. One-half ml. of a 10^{-4} dilution of an 18 hour broth culture was used as the inoculum.

5. Cardiopulmonary and renal studies. Standard 12 lead electrocardiograms were obtained on 29 patients and were obtained serially on all patients who were seriously ill. Venous pressure determinations were made on or shortly following admission in 23 patients and were studied serially in patients with symptoms or signs suggestive of congestive heart failure.

Arterial blood gas studies were made in eight patients by Dr. Daniel Lukas and his associates. Blood obtained from the brachial artery was analyzed for oxyhemoglobin saturation, oxygen binding capacity, and carbon dioxide content by the method of Van Slyke and Neill (9). Arterial pH was performed on the same specimens using a Cambridge research model pH meter. Serum carbon dioxide content was calculated from the nomogram of Van Slyke and Sendroy (10). Partial pressures of arterial carbon dioxide and serum bicarbonate were calculated from the Henderson-Hasselbalch equation using a solubility coefficient of 0.0301.

Blood plasma volumes were determined on four patients at the height of their pulmonary disease process by Dr. David Thompson and his associates using the T-1824 dye method of Chinard (11). T-1824 disappearance curves were also performed on two of these individuals. The dye disappearance curves obtained were compared to the values recorded in normal subjects by Noble and Gregerson (12). Phenolsulfonphthalein excretion and urea and inulin clearances were performed on selected patients.

6. Miscellaneous laboratory studies. Additional studies were performed on certain patients because of questions which arose during the course of this study.

1. Liver function tests were obtained on nine patients. These included determinations of total serum protein, albumin-globulin (A/G) ratios, total and fractionated serum bilirubin, cephalin flocculation, thymol turbidity and serum alkaline phosphatase.

2. Serum glutamic oxaloacetic transaminase levels were determined in 22 patients and were followed serially when abnormal results were encountered.

3. Studies on blood clotting were performed on five patients. These included standard bleeding and clotting times, clot retraction studies, plasma prothrombin determinations, capillary fragility tests and enumeration of blood platelets.

4. Serums were studied for the presence of cold agglutinins on from one to six occasions in 21 patients. Serum heterophile antibody was determined in 10 patients during the course of their illness.

5. Three patients developed signs and symptoms suggestive of central nervous system involvement. Electroencephalographic tracings were obtained, and studies of the cerebrospinal fluid, including attempts at virus isolation, were made in two of these patients.

6. Venous blood from two patients was analyzed for ketones and pyruvic acid by the methods of Bessman (13) and Friedemann and Haugen (14).

Values for these determinations in The New York Hospital laboratories are listed in Table I.

7. Postmortem studies. Ten patients died during the course of illness. An additional patient died with an acute myocardial infarction six weeks after discharge from the hospital. Complete postmortem studies were carried out in eight patients. In two in whom a complete autopsy was not permitted, specimens of lung were obtained by needle or incision for virologic, bacteriologic and microscopic examination.

TABLE I

Normal laboratory values (The New York Hospital)

Determination	Normal value
Blood urea nitrogen	8–15 mg. %
Serum carbon dioxide combining power	22–27 mM/L.
Serum sodium	132–143 mEq./L.
Serum potassium	3.5–5.3 mEq./L.
Serum chloride	97–106 mEq./L.
Serum glutamic oxaloacetic transaminase	0–35 units/ml./min.
Arterial blood pH	7.40 ± 0.02 units
Arterial oxyhemoglobin saturation	94–98%
Arterial carbon dioxide content	24–27 mM/L.
Arterial partial pressure carbon dioxide	39–42 mm. Hg
Arterial bicarbonate	22.8–25.8 mEq./L.
Blood pyruvic acid	60–125 μEq./L.
Blood ketones	10–80 μEq./L.
Corrected erythrocyte sedimentation rate (Wintrobe)	Males, 10 mm. in 1 hr. Females, 20 mm. in 1 hr.

RESULTS

General description of the patients studied

Thirty-two of the 33 patients included in this study were admitted to the hospital between Sept. 29 and Nov. 11, 1957. The remaining patient was admitted during January, 1958 (Figure 1). Patients ranged in age from 13 to 75 years. Approximately two-thirds (21 patients) were under 50 years of age. Eighteen were males and 15 were

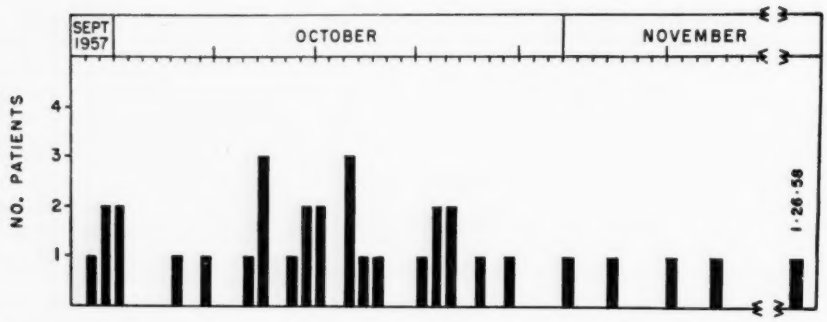

FIG. 1. DATES OF ADMISSION OF PATIENTS WITH PULMONARY COMPLICATIONS OF INFLUENZA

females. None of these patients had received influenza vaccination preceding the pandemic. Twenty-four patients had evidence of chronic disease or pregnancy antedating influenza. Symptoms suggesting the development of parenchymal lung involvement arose one to 14 days after the onset of initial influenzal symptoms.

In the following sections the four pulmonary syndromes observed are discussed separately along with the laboratory studies which aided in the characterization of each syndrome. Other laboratory studies which did not materially aid in differentiating these clinical entities are considered in the succeeding section. Subsequent sections review therapy, the course of the illnesses and studies on postmortem material. Selected case reports are included in the final section.

I. The clinical syndromes manifested by patients with influenza attended by signs of lower respiratory tract involvement

1. Influenza with physical signs of lower respiratory tract involvement without roentgenographic evidence of pneumonia

In three patients with influenza, chest findings developed which suggested parenchymal lung involvement in the absence of roentgenographic evidence of pulmonary infiltrates. All three of these individuals had evidence of underlying disease antedating the onset of influenza. Two patients had rheumatic heart disease. One patient with inactive pulmonary tuberculosis had known pulmonary hypertension as determined by previous cardiac catheterization studies.

Symptoms referable to the respiratory tract appeared within 48 hours of the onset of typical influenza symptoms. All three patients exhibited high fever and marked prostration; a cough which was productive of small amounts of blood tinged sputum appeared in two. Two patients noted pleuritic chest pain. Mild dyspnea was noted by all three patients.

Physical examination of the lungs revealed unilateral or bilateral crepitant and musical inspiratory rales accompanied by diminished or harsh breath sounds. Coarse rhonchi and occasional wheezes were heard in two of the three patients. Bilateral pleural friction rubs were noted in one patient. Signs of local consolidation were absent.

Total leukocyte counts were within the normal range in two patients; moderate leukocytosis was observed in one. Erythrocyte sedimentation rates were slightly elevated. Few bacteria were seen in sputum smears, and cultures from two patients demonstrated only normal pharyngeal flora. Occasional colonies of hemolytic *Staphylococcus aureus* and pneumococci were grown from sputum cultures obtained from the third patient. Blood cultures were sterile.

The Asian strain of influenza A virus was isolated from the throat washing of one of the two patients from whom washings were obtained. Serologic proof of influenza virus infection was obtained in all three. Symptoms and signs persisted from three days to several weeks. In each patient a complete recovery ensued on symptomatic treatment alone.

Comment. These three patients with influenza developed signs of lower pulmonary tract disease without X-ray evidence of pneumonia. There was no convincing evidence of bacterial infection. Recovery occurred without antimicrobial therapy.

These patients were believed to have bronchiolitis accompanying influenza. They are included to portray the full spectrum of pulmonary involvement witnessed during the epidemic, but are not considered further in the analysis of the more serious pulmonary complications of influenza.

2. Influenza complicated by secondary bacterial pneumonia

Fifteen patients had serologically established influenza followed by evidence of bacterial infection of the lung. These patients ranged in age from 13 to 72 years. Eight were males and seven were females.

Seven patients had evidence of pre-existing disease. Three had rheumatic heart disease with established mitral valve disease. Two patients were diabetic. One patient had bronchiectasis and pulmonary emphysema of long standing. One patient had progressive muscular dystrophy with involvement of the respiratory musculature. The remaining eight patients had no evidence of underlying disease prior to the onset of influenzal symptoms. One patient was in the third trimester of pregnancy. A summary of clinical data on these patients is recorded in Table II.

Clinical history. Ten of these 15 patients had a remarkably similar history. In each, the typical

TABLE II

Influenza complicated by bacterial pneumonia: Summary of clinical data

Patient, Age, Sex	Date of admission	Underlying disease*	Duration of illness prior to pulmonary symptoms	Pulmonary involvement‡							
				Symptoms				Signs			
				Chills	Productive cough	Chest pain	Dyspnea	Resp./ min.	Admission temp.	Cyanosis	Pulmonary findings§
			days†						°C.		
R. S. 40 F	9/29/57	RHD, MS, MI, AF	<1	0	0	0	++	28	40.0	0	Rales, RLL
N. Q. 21 M	9/30/57	0	4	0	+ Bloody	++	+	30	40.5	0	Consol., RLL
B. N. 52 F	10/1/57	RHD, MS, MI	<1	0	+ Bloody	0	++	30	39.8	0	Rales, harsh B.S., RLL, LLL
C. G. 59 M	10/4/57	0	3	+	+ Muco purulent	+	0	24	38.5	0	Consol., RLL, LLL
D. P. 13 F	10/11/57	0	<1	0	+ Purulent	0	0	26	39.6	0	Consol., LLL
M. M. 68 F	10/14/57	Mild diabetes	2	0	+ Bloody	0	0	22	38.8	0	Consol., RLL
R. L. 22 M	10/17/57	Muscular dystrophy	4	0	+ Bloody	+	++	22	39.6	±	Consol., RUL, RLL, LLL
P. R. 45 M	10/17/57	RHD, MS, MI	1	0	+ Bloody	++	0	28	38.6	0	Consol., RLL
N. M. 57 M	10/22/57	Chronic pulm. dis.	3	+	+ Muco purulent	0	0	20	40.0	0	Rales, LLL
A. L. 28 F	10/23/57	0	<1	+	+ Rusty	0	0	24	39.6	0	Consol., RLL
G. M. 42 F	10/24/57	Pregnant 8 months	6	0	+ Muco purulent	++	0	28	38.6	0	Rales, decr. B.S., RLL, LLL
H. W. 72 M	10/26/57	Mild diabetes	<1	0	+ Mucoid	++	0	40	39.2	+	Rales, decr. B.S., RLL, LLL
N. C. 24 F	11/1/57	0	14	+	+ Muco purulent	++	0	28	39.7	0	Consol., LLL
L. F. 63 M	11/4/57	0	4	0	+ Bloody	+	+	40	40.4	0	Consol., RUL, LLL
A. M. 40 F	11/11/57	0	3	0	+ Bloody	0	0	24	37.8	0	Consol., LLL

* RHD, rheumatic heart disease; MS, mitral stenosis; MI, mitral insufficiency; AF, auricular fibrillation; HCVD, hypertensive heart disease; ASCVD, arteriosclerotic heart disease; CO, coronary occlusion; AS, aortic stenosis; AI, aortic insufficiency.
† Where pulmonary symptoms blended with initial influenza symptoms the interval has been listed as <1.
‡ Cough, chest pain, dyspnea and cyanosis are graded in severity from 0 to +++.
§ RLL, right lower lung; LLL, left lower lung; RUL, right upper lung; LUL, left upper lung; B.S., breath sounds.

influenza symptoms had been followed by a definite period of improvement. Five patients felt strong enough to return to their usual occupations. Following a period of two to 14 days (average 3.8 days), there was a sudden reappearance of symptoms which suggested the onset of pulmonary infection. A shaking chill was experienced by four patients. Eight patients developed typical pleuritic chest pain and noted a marked increase in cough which was productive of bloody or purulent sputum.

Five of the 15 patients did not have a clear cut interval between the initial influenzal illness and symptoms of pulmonary involvement. In these patients pulmonary symptoms blended with the initial illness so that it was impossible to determine the time of onset of the bacterial complication.

Physical findings. All patients in this group appeared acutely ill. As noted in Table II, admission temperatures were above 39° C. in the majority, ranging from 38.6 to 40.5° C. Respiratory rates were only moderately increased, ranging from 20 to 30 respirations per minute in 13 of the 15 individuals. Cyanosis was uncommon and was observed in only two patients in association with pleuritic chest pain.

Examination of the chest in each instance revealed evidence of localized disease which corresponded with the abnormalities found on roentgenographic examination. Ten patients had classic local signs of consolidation including dullness to percussion, increased fremitus, bronchial breathing, egophony and bronchophony. Five patients had localized rales, rhonchi and abnormal breath sounds without signs of classic consolidation. Single lobe involvement was observed in nine patients and bilobar disease in five. One patient had consolidation of three lobes. Signs of diffuse pulmonary involvement were not observed in these patients.

Laboratory studies. Total leukocyte counts obtained at the time of admission ranged from 3,300 to 24,600 white blood cells per cu. mm. A leukocytosis of 10,000 or more was present in 11. Polymorphonuclear leukocytes predominated, and an increase in band forms was noted in every case. Admission white blood cell counts and the observed range of total leukocyte and absolute granulocyte counts during the acute illness are portrayed in Figure 2. Ten of the 15 patients showed elevated erythrocyte sedimentation rates at the time of their admission.

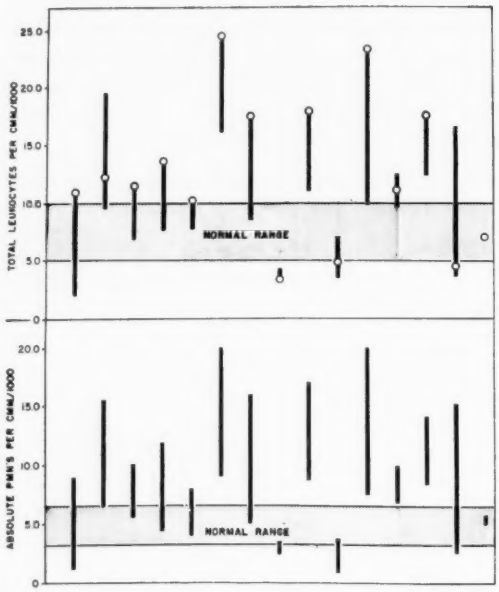

FIG. 2. TOTAL LEUKOCYTE AND POLYMORPHONUCLEAR LEUKOCYTE COUNTS IN PATIENTS WITH INFLUENZA COMPLICATED BY BACTERIAL PNEUMONIA

Admission white blood cell counts are indicated by the open circles; the vertical bars indicate the range in values during the acute illness.

Chest roentgenograms. In all 15 patients chest roentgenograms revealed evidence of lobar or lobular involvement of the type commonly observed with bacterial pneumonia (see Figures 12, 14, and 15 accompanying the case reports). In 10, true lobar distribution of the pulmonary infiltrate was observed. Infiltrates were dense and localized and halted abruptly at fissure lines. One patient with staphylococcal pneumonia developed cavitation within the involved lobe. Empyema or the development of significant amounts of pleural fluid was not observed. In no patient in this group did X-ray films reveal extension of the process following the institution of antimicrobial therapy.

Bacteriologic findings. Sputum smears contained many polymorphonuclear leukocytes and large numbers of bacteria. A heavy growth of pneumococci was obtained in sputum and throat cultures from eight patients. Coagulase positive *Staphylococcus aureus* was isolated as the predomi-

nating microorganism, or in pure culture from the sputum of three patients. *Hemophilus influenzae* was the only microorganism recovered from the sputum of one patient.

Sputum cultures from three patients revealed mixed bacterial growth. Both pneumococci and staphylococci were isolated in large numbers from the sputum of one patient. Coagulase positive staphylococci and *Hemophilus influenzae* were obtained from another, and coagulase positive staphylococci, *Aerobacter aerogenes,* and *Candida albicans* were grown from sputum cultures of one patient who acquired her disease within the hospital (Table III).

Virologic studies. Throat washings obtained from 13 patients on the third to the thirty-fifth day of illness were studied for the presence of influenza virus by methods detailed elsewhere (*7*). Influenza virus was not recovered from any patient in this group.

Serologic studies. Both acute sera and convalescent sera were available in eight patients. In these eight individuals, a fourfold or greater rise in complement fixing antibody titer was demon-

TABLE III

Influenza complicated by bacterial pneumonia: Summary of bacteriologic and virologic data

			Influenza virus studies								
						Complement fixation					
	Bacteriologic studies		Virus isolation			Day of illness serum obtained		Reciprocal of CF titer			
Patient	Specimen	Cultural findings	Specimen	Day of illness obtained	Result	1	2	1	2	Treatment†	Outcome
R. S.	Throat blood	Pneumococcus 0	TW*	3	0	6	19	16	4,096	EM Te	Recov.
N. Q.	Sputum blood	Hem. *Staph. aureus* 0					22		512	Pen EM SM	Recov.
B. N.	Sputum Blood	Few hem. *Staph. aureus* *A. aerogenes* Monilia 0	TW	15	0	3	17	16	1,024	Pen EM CM SM	Expired 37 days
C. G.	Sputum Blood	Hem. *Staph. aureus* *H. influenzae* 0	TW	12	0	14	24	512	1,024	Pen EM CM SM	Recov.
D. P.	Sputum Blood	Pneumococcus VIII 0	TW	7	0	9	16	32	512	Pen	Recov.
M. M.	Sputum Blood	Pneumococcus III 0	TW	6	0	6	16	32	128	Pen	Recov.
R. L.	Throat Blood	Pneumococcus III 0	TW	7	0	7	18	512	1,024	Pen EM SM	Recov.
P. R.	Sputum Blood	Pneumococcus Hem. *Staph. aureus* 0	TW	4	0	4	19	8	128	Pen EM SM	Recov.
N. M.	Sputum Blood	Pneumococcus 0	TW	6	0	6	13	64	2,048	Pen	Recov.
A. L.	Sputum Blood	Pneumococcus 0	TW	1	0	1	8	16	256	Pen	Recov.
G. M.	Sputum Blood	*H. influenzae* 0	TW	13	0	12	30	512	1,024	CM SM	Recov.
H. W.	Sputum Blood	Hem. *Staph. aureus* 0	TW	35	0		35		512	EM CM SM	Recov.
N. C.	Sputum Blood	Pneumococcus 0				19	30	128	64	Pen EM SM	Recov.
L. F.	Sputum Blood	Hem. *Staph. aureus* 0	TW	9	0	11	26	256	512	Pen EM SM	Recov.
A. M.	Sputum Blood	Pneumococcus 0	TW	7	0	7	15	128	4,096	Pen	Recov.

* TW, Throat washings.
† EM, Erythromycin; Te, Tetracycline; Pen, Penicillin; SM, Streptomycin; CM, Chloramphenicol.

TABLE IV

Primary influenza virus pneumonia: Summary of clinical data

Patient, Age, Sex	Date of admission	Underlying disease*	Duration of illness prior to pulmonary symptoms†	Pulmonary involvement‡							
				Symptoms				Signs			
				Chills	Productive cough	Chest pain	Dyspnea	Resp./ min.	Admission temp.	Cyanosis	Pulmonary findings*
			days						°C.		
G. H. 49 M	10/5/57	RHD, AF, MS, MI	<1	0	++ bloody	0	+++ severe	40	40.2	++	Bilat. basilar insp. rales
C. P. 47 M	10/11/57	RHD, MI	<1	+	++ mucoid	0	+++ severe	38	39.5	+++	Diffuse bilat. wheezing, rales
A. B. 45 M	10/13/57	RHD, MI, AS, AI	<1	+	++ bloody	0	+++ severe	36	40.0	+++	Diffuse harsh B.S., Insp. rales, RLL, LLL
P. B. 45 M	10/14/57	RHD, AF, MS	<1	0	++ bloody	0	+++ severe	42	40.4	+++	Diffuse suppression, B.S., rales, RLL, LLL
J. B. 75 F	10/15/57	HCVD	<1	?	++ bloody	?	+++ severe	54	39.2	+++	Diffuse bilat. wheezing, rales
A. C. 66 M	10/19/57	ASCVD C.O.	<1	+	+ mucoid	0	+++ severe	40	39.4	++	Harsh B.S., diffuse bilat. rales

* Abbreviations are the same as those used in Table II.
† Where pulmonary symptoms blended with initial influenza symptoms the interval has been listed as <1.
‡ Cough, chest pain, dyspnea and cyanosis graded in severity from 0 to +++.

strated with the Asian strain of influenza A virus. Seven patients were admitted seven to 35 days after the onset of influenza symptoms. In these seven patients initial complement fixing antibody titers of 1 : 128 or greater were demonstrated. A summary of bacteriologic and virologic data appears in Table III.

3. Primary influenza virus pneumonia

Six patients in the present series manifested a diffuse pulmonary syndrome which was believed to represent primary influenza virus infection without secondary bacterial infection.

These patients constituted a relatively uniform group. All six patients had established heart disease. Five of the six were males. Four males between the ages of 45 and 49 years had antecedent rheumatic heart disease. Severe mitral valve disease was present in three, combined mitral and aortic valvular disease in one. A 66 year old male had arteriosclerotic heart disease with a history of two previous myocardial infarctions. The single female, a 75 year old woman, had known hypertensive cardiovascular disease.

Clinical history. In the five patients who gave an adequate clinical history, a remarkably uniform sequence of events had occurred. Each patient had experienced an illness characteristic of influenza with chilly sensations, high fever, scratchy sore throat, a dry, nonproductive cough, headache, muscular aching and prostration. Over the next 24 hours increasing respiratory distress caused each patient to seek hospitalization. Thus each of these patients was admitted within 36 hours of the onset of the initial symptoms of influenza.

In three patients increasing dyspnea was associated with a distressing cough productive of bloody sputum. Three patients had sustained one or more shaking chills. In no instance was pleural pain or purulent sputum noted. Certain clinical features of this illness are summarized in Table IV.

Physical examination. On admission each of these patients appeared acutely and seriously ill. Admission temperatures ranged from 39.2 to 40.4° C. Respiratory distress was profound with respiratory rates of 36 to 54 per minute. Extreme dyspnea made speech difficult in all instances. Moderate to profound cyanosis was present in all six patients. In none was shock apparent at the time of admission. Venous distention, hepatomegaly and peripheral edema were not present.

Examination of the lungs revealed findings which were quite different from those noted in

the group with secondary bacterial pneumonia. In general, findings were diffuse, and in no instance were focal signs of consolidation (dullness to percusion, bronchial breath sounds, egophony, bronchophony) detected.

In three patients the only physical findings on admission consisted of fine inspiratory rales accompanied by scattered inspiratory wheezes and expiratory prolongation over the lower lung fields. As the disease progressed, physical findings became more extensive and frequently varied from hour to hour. As dyspnea and cyanosis increased, air exchange became progressively difficult. At times, increasing suppression of breath sounds was evident over the lower lung fields posteriorly, spreading gradually to involve the entire chest. At other times, patients were noted to develop increasing expiratory difficulties with diffuse inspiratory rales and prominent expiratory wheezing.

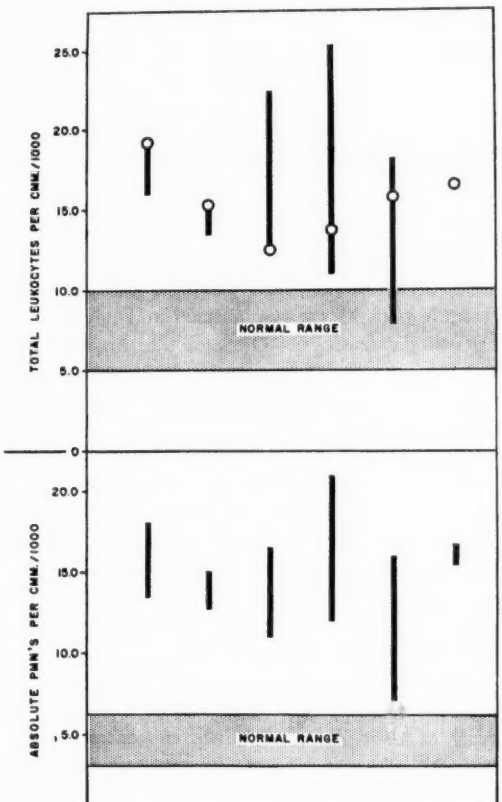

FIG. 3. TOTAL LEUKOCYTE AND POLYMORPHONUCLEAR LEUKOCYTE COUNTS IN PATIENTS WITH PRIMARY INFLUENZA VIRUS PNEUMONIA

Admission white blood cell counts are indicated by the open circles; the vertical bars indicate the range in values during the acute illness.

In the terminal stages of the disease, findings on physical examination were similar in all patients. Harsh suppressed breath sounds were heard throughout both lung fields. Generalized sticky inspiratory rales were evident. Coarse bubbling rales and grating rhonchi obscured expiratory breath sounds, and the expiratory phase was markedly prolonged. At this stage dyspnea was unbearable, and respirations were rapid and gasping in nature. Marked agitation was common, and intense cyanosis was observed in all patients.

Routine laboratory studies. Total leukocyte counts on admission ranged from 13,700 to 19,200 cells per cu. mm., and a marked leukocytosis was maintained throughout the acute illness. In every case there was a striking preponderance of polymorphonuclear leukocytes and an increase in immature band forms (Figure 3). Erythrocyte sedimentation rates were within normal limits in four of five patients studied. These are depicted in Figure 4 and are contrasted with the elevated sedimentation rates found in the majority of patients with secondary bacterial pneumonia or concomitant viral and bacterial pneumonia.

Chest roentgenograms. Chest roentgenograms revealed diffuse infiltrates which mimicked the roentgenographic picture of cardiac pulmonary edema. In general, a fanning perihilar infiltrate of a diffuse and nodular nature was noted. The infiltrate was more striking toward the lung bases and bronchovascular markings were increased. Two patients showed small loculated interlobar effusions. In one of these patients the effusion was present prior to admission. Typical X-ray pictures are presented in Figures 17, 19, 20 and 21 which accompany the case reports.

Bacteriologic findings. In none of the six patients were significant numbers of pathogenic bacteria isolated from the sputum. Stained smears of sputum revealed only a few polymorphonuclear leukocytes and scant bacteria in three patients. In the remaining three patients polymorphonuclear leukocytes were noted in stained sputum preparations, but only rarely could bacteria be found. One or more sputum and pharyngeal cultures

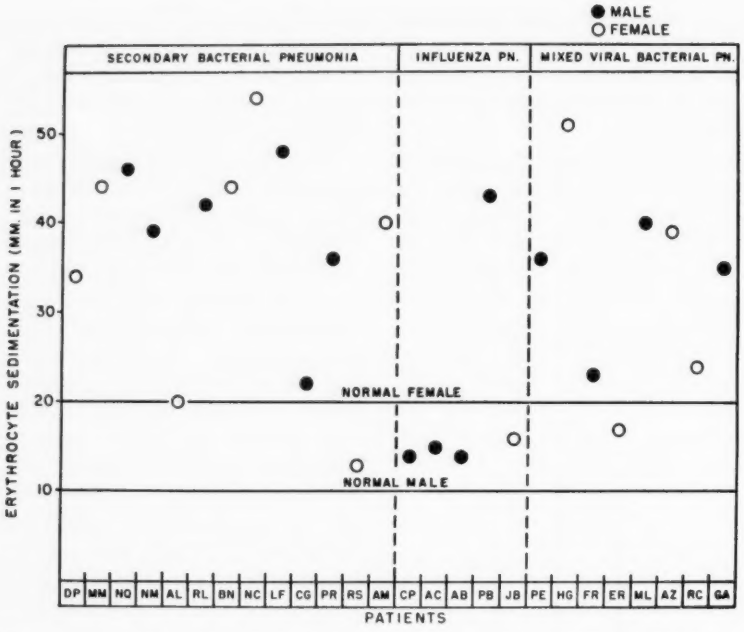

FIG. 4. ADMISSION ERYTHROCYTE SEDIMENTATION RATES (WINTROBE METHOD) IN PATIENTS WITH PULMONARY COMPLICATIONS OF INFLUENZA

taken prior to the institution of therapy demonstrated only normal throat inhabitants (alpha and gamma strepococci, *Neisseria* and diptheroids) in two patients. An occasional colony of pneumococci was noted in sputum cultures obtained from four patients but normal flora predominated. A few colonies of *Hemophilus influenzae* were also isolated from the sputum from two of these four patients.

Mice injected with sputum remained healthy, and bacteria could not be isolated on subsequent culture of the peritoneal cavities. Blood cultures taken on one or more occasions from each patient were sterile.

Extensive bacteriologic studies were performed on emulsions of lung tissue obtained from the five patients who died. Multiple cultures from four patients were sterile on incubation for 10 days. The fifth patient dying on the eleventh day of illness had a clear-cut episode of aspiration two days before death. Fungi and bacteria could be seen on microscopic section surrounding aspirated food and muscle fibers within the bronchi. Moderate numbers of *Candida albicans* and *Escherichia coli* were obtained on lung cultures from this patient. Bacteriologic and virologic data on this group of patients are summarized in Table V.

Virologic findings. Three of the throat washings obtained from four patients on the second and third day of illness yielded the Asian strain of influenza A virus. Influenza virus was not isolated from the throat washing of Patient P. B., the only patient in this group who survived. A significant rise in complement fixing antibody was demonstrated in this patient. Attempts at virus recovery from concentrated urine specimens of two patients were unsuccessful. Cerebrospinal fluid obtained from one patient on the fifth day of illness did not yield influenza virus.

Influenza virus was isolated from the lungs of the four patients who died within five days of onset of their disease. Virus concentrations of 10^2, 10^4, $10^{5.3}$ and $10^{5.7}$ 50 per cent egg infectious doses (EID_{50}) per Gm. of lung were found in these specimens (Table V).

4. Combined influenza virus and bacterial pneumonia

Nine patients in the present series had an illness accompanied by clinical and laboratory fea-

TABLE V

Primary influenza virus pneumonia: Summary of bacteriologic and virologic data

Patient	Bacteriologic studies		Influence virus studies							Treatment†	Outcome	Postmortem bacteriology (lung)	Post-mortem influenza virus isolation conc./Gm. lung‡
			Virus isolation			Complement fixation							
						Day of illness serum obtained		Reciprocal of CF titer					
	Specimen	Cultural findings	Specimen	Day of illness obtained	Result	1	2	1	2				
G. H.	Sputun.	NF*								O_2, Pen, EM, SM, CM, Hydrocortisone Levarterenol	Died 5 days	Sterile	$+++$ $10^{5.3}$
	Blood	Rare pneumococcus 0, 0											
C. P.	Sputum	NF Rare pneumococcus Pseudomonas, Proteus								O_2, Pen, SM, EM, Cpd. F, Levarterenol	Died 3 days	Sterile	$+++$ $10^{5.7}$
	Blood	0, 0											
A. B.	Sputum	NF	TW*	3	+					O_2, Pen, EM, SM, Hydrocortisone Prednisone	Died 4 days	Sterile	$+++$ 10^{4}
		Few *H. influenzae* Pneumococcus	Urine	3	0								
	Blood	0, 0											
P. B.	Sputum	NF Rare pneumococcus *H. influenzae*	TW	2	0	3	13	32	512	O_2, Pen, EM, SM, Prednisone, Acetazoleamide	Recovery		
	Blood	0, 0, 0											
J. B.	Sputum	NF	TW	3	+					O_2, Pen, SM, CM, Hydrocortisone Levarterenol	Died 11 days	C. albicans E. coli	0
	Blood	0, 0	Urine	3	0								
			CSF*	5	0								
A. C.	Sputum	NF	TW	3	+					O_2, Pen, EM, SM, Levarterenol	Died 4 days	Sterile	+ 10^{2}
	Blood	0											

* NF, normal flora (alpha and gamma streptococci, diphtheroids, neisseria); TW, throat washings; CSF, cerebrospinal fluid.
† Pen, penicillin; EM, erythromycin; SM, streptomycin; CM, chloramphenicol.
‡ Concentrations of virus recorded in 50 per cent egg infectious doses (EID_{50}).

tures which suggested that both influenza virus and specific bacterial pathogens were responsible for their lower respiratory tract disease. These patients ranged in age from 21 to 61 years. Four were males and five were females. There was evidence of antecedent chronic disease in six. Three patients had rheumatic heart disease with mitral valve involvement; one of these three patients was also in the sixth month of pregnancy. Two patients had arteriosclerotic cardiovascular disease with previously documented myocardial infarctions. One patient had multiple myeloma. One of the three patients without evidence of underlying disease was in the eighth month of pregnancy.

Clinical history. Eight patients in whom a reliable history could be obtained gave a story of typical influenza. Four patients noted a period of improvement lasting two to four days before symptoms suggesting lower respiratory tract infection appeared. In four patients the symptoms of lower pulmonary tract disease blended with the original influenzal symptoms, and it was impossible to determine the point at which the pulmonary involvement had occurred. Severe dyspnea similar to that noted by patients with the syndrome of primary influenza virus pneumonia caused the admission of each patient.

Seven patients had noted cough which was productive of grossly bloody sputum in four and was purulent in three. Shaking chills were noted by five patients. Four patients noted pleuritic chest pain.

Physical findings. All nine patients appeared seriously ill at the time of admission. Admission temperatures ranged from 38.6 to 40.4° C. Severe respiratory distress was present in all, and respiratory rates above 36 respirations per minute were present in seven individuals. Cyanosis was noted in eight patients. Two patients with preexisting heart disease showed signs of congestive failure. In the remaining seven patients, no venous distention or other signs of cardiac failure were noted.

Examination of the lungs revealed variable

TABLE VI

Concomitant influenza virus and bacterial pneumonia: Summary of clinical data

Patient, Age, Sex	Date of admission	Underlying disease*	Duration of illness prior to pulmonary symptoms†	Pulmonary involvement‡							
				Symptoms				Signs			
				Chills	Productive cough	Chest pain	Dyspnea	Resp./ min.	Admission temp.	Cyanosis	Pulmonary findings*
			days						°C.		
H. G. 32 F	10/11/57	0 (pregnant 8 mos.)	4	+	+ purulent	+	+++ severe	60	38.6	++	Consol., RLL Diffuse insp. rales
P. E. 38 M	10/15/57	RHD, MS, MI, AS, AI	1	+	+ bloody	0	+++ severe	44	39.5	++	Consol., RLL Diffuse wheeze, harsh B.S.
E. R. 35 F	10/17/57	RHD, MS (pregnant 6 mos.)	1	+	+ bloody	+	++	44	39.8	+	Consol., LLL
G. A. 57 M	10/18/57	ASCVD old C.O.	1	+	+ bloody	0	++	34	39	0	Bilat. basilar rales
R. C. 31 F	10/23/57	RHD, MS	2	+	+ purulent	+	++	28	40	+	Consol., RML Insp. rales, RLL, LLL
M. L. 66 M	10/24/57	ASCVD old C.O.	1	0	+ muco purulent	0	+++ severe	40	39.6	+	Diffuse insp., exp. rales
F. R. 53 M	10/28/57	Multiple myeloma	? 1–4	Unreliable history				38	39.2	+++	Rales, RLL, LLL
A. Z. 21 F	11/8/57	0	3	0	+ sanguino purulent	++	+++ severe	60	40.3	+++	Consol., RLL, LLL Diffuse rales, wheezes, harsh B.S.
B. P. 61 F	1/26/58	0	3	0	++ bloody	0	+++ severe	60	40.4	+++	Consol., LLL Diffuse rales, wheezes, rhonchi

* Abbreviations are the same as those used in Table II.
† Where pulmonary symptoms blended with initial influenza symptoms the interval has been listed as <1.
‡ Cough, chest pain, dyspnea, and cyanosis graded in severity from 0 to +++.

findings. Six patients had evidence of local consolidation of one or more lobes. Five of these patients had, in addition, diffuse signs which included inspiratory rales, prolongation of expiration, wheezing and diminished or harsh breath sounds. These findings were similar to the pulmonary findings noted in the patients described previously with primary influenza virus pneumonia. Three patients had diffuse pulmonary findings without signs of focal consolidation. Clinical data are summarized in Table VI.

Routine laboratory studies. Total leukocyte counts obtained on admission ranged from 1,400 to 21,000 leukocytes per cu. mm. Six of the nine patients showed a marked leukocytosis with an increase in immature leukocytes. Two patients showed a striking leukopenia and granulocytopenia. In both of these patients *Staphylococcus aureus* was the bacterial pathogen and both subsequently died of their illness. Total leukocyte and polymorphonuclear leukocyte counts obtained during the course of the acute illness are pictured in Figure 5. Erythrocyte sedimentation rates ranged from 17 to 51 mm. per hour (Figure 3), and were significantly elevated in five patients.

Chest roentgenograms. Seven patients showed both local lobar infiltrates and bilateral nodular perihilar infiltrates on admission chest roentgenograms. Diffuse perihilar infiltrates similar to those seen in the group believed to have primary influenza virus pneumonia were observed in two patients. Typical roentgenograms are pictured in Figures 24, 26, 27 and 29 which accompany the case histories.

Bacteriologic findings. Examination of sputum smears obtained on admission commonly revealed large numbers of polymorphonuclear leukocytes, even in patients with leukopenia. Large numbers of microorganisms could be seen in sputum smears. On sputum culture, coagulase positive *Staphylococcus aureus* was isolated in pure culture from three patients. Pneumococci in relatively pure culture were isolated from three. Sputum cultures resulted in growth of both pneumococci and hemolytic *Staphylococcus aureus* in three instances. A few colonies of *Hemophilus influenzae* were isolated from the sputum culture of one patient in addition to staphylococci and pneumococci. Repeated blood cultures were sterile in all patients. Postmortem cultures

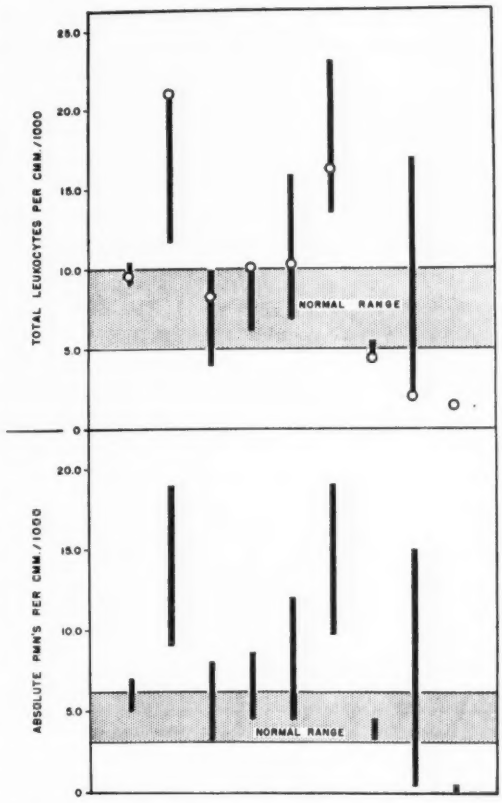

FIG. 5. TOTAL LEUKOCYTE AND POLYMORPHONUCLEAR LEUKOCYTE COUNTS IN PATIENTS WITH CONCOMITANT INFLUENZA VIRUS AND BACTERIAL PNEUMONIA

Admission white blood cell counts are indicated by the open circles; the vertical bars indicate the range in values during the acute illness.

were obtained on all four patients who died. Lung emulsions from two patients yielded coagulase positive staphylococci in concentrations of 10^8 microorganisms per ml. The lungs of one patient dying 53 days after the onset of influenza with an unusual myocarditis and pulmonary arteritis showed a few colonies of coagulase positive staphylococci, *Candida albicans* and *E. coli*, but there was no histologic evidence of significant bacterial infection. Specimens of lung obtained from one patient dying on the sixth hospital day were sterile on culture. This patient had had large numbers of pneumococci isolated from initial sputum cultures.

Virologic studies. Influenza A virus of the

TABLE VII

Concomitant influenza virus and bacterial pneumonia: Summary of bacteriologic and virologic data

Patient	Bacteriologic studies		Influenza virus studies							Treatment†	Outcome	Postmortem bacteriology (lung)	Post-mortem influenza virus isolation conc./Gm. lung§
			Virus isolation			Complement fixation							
						Day of illness serum obtained		Reciprocal of CF titer					
	Specimen	Cultural findings	Specimen*	Day of illness obtained	Result	1	2	1	2				
H. G.	Sputum Blood	Hem. *Staph. aureus* 0	TW Urine	7 8	0 0	8	17	256	2048	O_2, Pen, EM, SM	Recovery		
P. E.	Sputum Blood	Pneumococcus Hem. *Staph. aureus* 0	TW CSF	3 6	+ 0	3	33	anti comp	1024	O_2, Pen, EM, SM, Cpd. F, Prednisone	Recovery		
E. R.	Sputum Blood	Pneumococcus 0	TW	1	+	2	14	128	512	O_2, Pen, SM, CM, Prednisone	Recovery		
G. A.	Sputum Blood	Pneumococcus Hem. *Staph. aureus* Few *H. influenzae* 0	TW	3	+	6	16	16	512	O_2, Pen, EM, SM, Acetazoleamide	Recovery‡		
R. C.	Sputum Blood	Pneumococcus Hem. *Staph. aureus* 0	TW	5	0	6	38	256	2048	O_2, Pen, EM, CM, Prednisone	Died 53 days	Few colonies Hem. *Staph. aureus*, *Cand. albicans*, *E. coli*	0
M. L.	Sputum Blood	Pneumococcus 0	TW Blood	3 3	+ 0	3	23	8	256	O_2, Pen, SM, CM, Prednisone	Recovery		
F. R.	Sputum Blood	Pneumococcus 0	TW Blood	1–4 1–4	+ 0					O_2, Pen, CM, SM, Cpd. F, Cortisone	Died ? 6 to 10 days	Sterile	$+$ 10^2
A. Z.	Sputum Blood	Hem. *Staph. aureus* 0	TW Blood Urine	4 4 4	+ 0 0					O_2, EM, SM, CM, Cpd. F, Prednisone, Acetazoleamide, Levarterenol	Died 10 days	Hem. *Staph. aureus* 5×10^8/Gm.	$+$ 10^2
B. P.	Sputum Blood	Hem. *Staph. aureus* 0	TW	5	+					O_2, Pen, SM, CM, Cpd. F, Levarterenol	Died 5 days	Hem. *Staph. aureus* 4×10^8/Gm.	$+$ $10^{2.7}$

* TW, throat washings; CSF, cerebrospinal fluid.
† Pen, penicillin; EM, erythromycin; SM, streptomycin; CM, chloramphenicol.
‡ Died 12/16/57 of acute myocardial infarction.
§ Concentrations of virus recorded in 50 per cent egg infectious doses (EID_{50}).

Asian strain was recovered from seven of nine patients in whom throat washings were obtained prior to the fourth day of illness. The two throat washings negative for influenza virus were obtained on the fifth and seventh days of disease.

Blood specimens obtained from three patients were inoculated into chick embryos without recovery of influenza virus. Concentrated urine specimens from two patients and one cerebrospinal fluid obtained on the sixth day of illness from a patient manifesting central nervous system signs likewise failed to yield influenza virus (7).

Paired serologic specimens obtained on six patients showed a fourfold or greater increase in complement fixing antibody to the Japan strain of influenza A virus.

At autopsy, low titers of influenza virus were demonstrated in the lungs of three patients, all of whom died prior to the ninth day of their illness. No influenza virus was recovered from the patient who died 53 days after the onset of influenza. Bacteriologic and virologic data on these cases are summarized in Table VII.

II. Special studies performed on patients with influenza and pneumonia

1. Cardiac studies

Many patients in the current series had evidence of underlying heart disease. Because of this high incidence of pre-existing cardiac abnormalities, and reports of myocarditis associated with influenza (15, 16), a careful evaluation of cardiac function was made in all patients. Thirteen of the 15 patients with localized secondary bacterial pneumonia had no clinical or laboratory findings suggestive of cardiac involvement. The two remaining patients had severe rheumatic heart disease, and evidence of congestive heart failure accompanied their pneumonia. Dyspnea, orthopnea and moderately elevated venous pressures of 140 and 165 mm. saline were noted in these two patients.

In contrast, all of the patients who presented the diffuse pulmonary syndromes with either primary influenza virus pneumonia or combined bacterial and viral pneumonia showed striking dyspnea, orthopnea and cyanosis. Pulmonary findings were usually compatible with left heart failure. There were, however, certain features which suggested that the acute pulmonary disease observed was secondary to pulmonary infection rather than cardiac failure:

a. First, the high fever, leukocytosis and profound systemic symptoms suggested that an acute infectious process was present.

b. Second, cardiac findings suggesting serious myocardial involvement, such as heart sounds of poor quality, diastolic gallop, pulsus alternans or narrowing pulse pressure, were not observed.

c. Third, signs of right heart failure were consistently absent in 13 of the 15 patients with diffuse pulmonary syndromes, despite the presence of significant pre-existing heart disease in 11 of these patients. Venous pressure determinations were transiently elevated (120 mm. and 150 mm. saline) in two patients who were agitated at the time of study. The remaining venous pressures in these 13 patients were consistently normal. Two patients with concomitant influenza virus and bacterial pneumonia had definite signs of congestive heart failure which included peripheral edema, distended neck veins, hepatomegaly and elevated venous pressures. A summary of venous pressure determinations is recorded in Figure 6.

d. Blood volume studies. Determinations of blood and plasma volume were made on four patients at the height of their disease process. Blood volumes and plasma volumes were within predicted normal limits in three patients. A minimally elevated blood volume (118 per cent) was noted in one patient. Because of evidence of progressive fluid accumulation in the lungs, T-1824 disappearance curves were also performed on two of these patients in an attempt to detect whether a generalized increase in vascular permeability was present. In both instances disappearance curves were normal. These data are summarized in Table VIII.

e. Electrocardiographic studies. Electrocardiograms were obtained on one or more occasions in 12 of 15 patients with secondary bacterial pneumonia. In four instances, electrocardiographic abnormalities were noted. Three patients with rheumatic heart disease showed electrocardiographic changes compatible with their underlying cardiac lesions without other abnormalities. One patient, a 72 year old male with mild diabetes, showed abnormal T waves and RT segments suggestive of

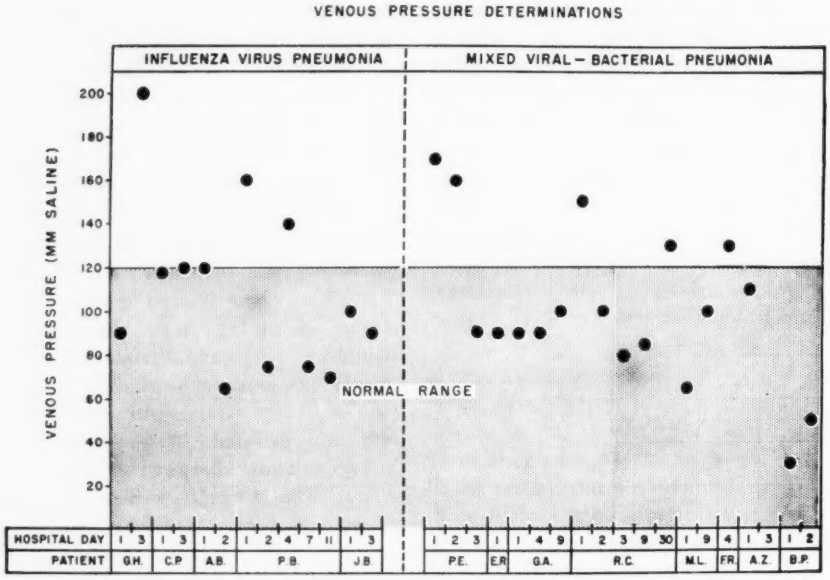

FIG. 6. VENOUS PRESSURE DETERMINATIONS IN PATIENTS WITH PRIMARY INFLUENZA OR VIRUS PNEUMONIA, CONCOMITANT INFLUENZA VIRUS AND BACTERIAL PNEUMONIA

Pressures recorded in mm. saline above the level of the right auricle.

coronary artery disease. No changes suggestive of myocarditis were noted.

Serial electrocardiograms were obtained on 14 of the 15 patients with diffuse pulmonary involvement. One patient without previous known heart disease (B. P.) showed electrocardiographic changes suggesting early left ventricular hypertrophy. Patients with underlying heart disease showed electrocardiographic abnormalities compatible with their basic heart disease. These abnormalities persisted without change throughout the course of illness. One patient with rheumatic heart disease (C. P.) had bouts of ventricular tachycardia a few hours preceding death. The remaining patients without antecedent heart disease had normal electrocardiograms. Again,

TABLE VIII

Blood volume studies and T-1824 disappearance rates in four patients with pneumonia complicating influenza *

Patient, Age, Sex	Type of pneumonia	Day of illness	Plasma volume	Predicted plasma volume	Plasma vol. as % of predicted norm.	Hematocrit†	Blood volume	Blood vol. as % of predicted norm.	T-1824 disappearance (% in 1st hour)‡
			L.	*L.*		%	*L.*		
A. B. 45 M	Primary virus	3	3.20	3.11	103	40	5.34	95	
P. B. 45 M	Primary virus	2	3.33	3.26	102	40.1	5.56	95.1	5.84
J. B. 75 F	Primary virus	3	2.89	2.54	114	39.6	4.78	118	4.45
P. E. 38 M	Mixed viral-bacterial	2	3.34	3.15	106	45.6	6.13	108	
		3	3.51	3.15	111	39.5	5.80	102	

* T-1824 technique (11).
† Corrected for 4 per cent trapped plasma.
‡ Average T-1824 disappearance in normal males, 5.2 per cent ± 1.65 (12).

TABLE IX

Blood gas data on eight patients with influenza virus pneumonia or combined influenza virus and bacterial pneumonia

Patient, Age, Sex, Underlying disease*	Day of illness	Status of disease	Conditions of test	Arterial blood gas studies				
				pH units	O_2 sat. %	pCO_2 *mm. Hg*	CO_2 content *mM/L.*	HCO_3 *mEq./L.*
			Primary influenza virus pneumonia					
P. B. 45 M RHD MS–MI	3	Day of admission, critically ill, cyanotic, tachypneic	Off O_2	7.43	84.8	45	30.3	28.9
	4	Apparent improvement, less respiratory distress	Off O_2	7.41	91.8	51	33.2	31.7
	5	Condition deteriorating, increasing respiratory distress	IPPB† 100% O_2	7.25	92.8	51	23.06	21.6
	6	No significant change	Off O_2	7.31	83.8	76	39.43	37.2
	7	Initial improvement, beginning diuresis	IPPB 100% O_2 4 hours post acetazoleamide	7.38(v)‡		53§		
	8	Marked diuresis, improvement	Off O_2 Acetazoleamide, 1 Gm. daily	7.30	92.9	47	23.62	22.61
J. B. 75 F HCVD	3	Day after admission, comatose, tachypneic, cyanotic	Off O_2	7.21	46.2	67	28	26
	3	30 minutes later	Positive pressure mask 100% O_2	7.16	91.0	76	28.7	26.4
	3	30 minutes later	IPPB 100% O_2	7.25	96.5	47	21.6	20.2
	4	Coma lighter, respirations less labored	Off O_2	7.36	73.5	39	22.4	21.2
	4	30 minutes later	IPPB 100% O_2	7.23	91.5	54	23.6	21.9
	5	Same as day 4	Off O_2	7.14	69.9	60	22	20.1
	5	30 minutes later	IPPB 40% O_2	7.13	94.7	63	22.4	20.6
	8	Slight improvement, lungs clearing, coma continuing	Off O_2	7.34	74.2	37	20.6	19.5
			Concomitant influenza and bacterial pneumonia					
P. E. 38 M RHD MS–MI AS–AI	3	Day after admission, critically ill, cyanotic, tachypneic	Off O_2	7.46	80.1	37	26.8	25.7
	4	No change	IPPB 100% O_2	7.40	87.7	46.4	29.3	27.9
	8	Steady improvement	Off O_2	7.48	85.4	42.0	31.0	29.7
	13	Steady improvement	Off O_2	7.38	92.6	46.0	27.7	26.3
	16	Steady improvement	Off O_2	7.36	92.2	52.0	30.0	28.4
A. Z. 21 F	4	Day of admission, critically ill, cyanotic, tachypneic	Off O_2	7.42	71.1	25	16.8	16.1
	4	30 minutes later	Positive pressure mask 100% O_2	7.40	82.6	28	17.5	16.7
	4	30 minutes later	IPPB 100% O_2	7.36	93.9	34	19.4	18.5
	8	Semi comatose, obstructive respirations	IPPB 100% O_2	7.22	97.2	78	33.2	30.8
	9	Comatose, obstructive respirations	IPPB 100% O_2 Post acetazoleamide	7.44	97	39	27.1	25.9
F. R. 53 M Multiple myeloma	? 5–9	Increasing respiratory distress, cyanosis	Off O_2	7.25	50.5	58	26.4	24.6
	? 5–9	30 minutes later	IPPB 100% O_2	7.23	91.5	62	27.0	25.0
M. L. 66 M ASCVD	3	Day of admission, critically ill, cyanotic, tachypneic	Off O_2	7.28(v)‡	80.3	41§	20.8§	19.6§
	7	Moderate improvement	Off O_2	7.45	90.0	37	25.0	26.1
B. P. 61 F	5	Day after admission, critically ill, cyanotic, tachypneic	Off O_2	7.18	70.5	27	10.5	9.6
G. A. 57 M ASCVD	11	Improving, respiratory distress disappearing	Off O_2	7.23(v)‡	93	55.1§	23.1§	23.4§

* Abbreviations the same as those used in Table II.
† IPPB, Intermittent Positive Pressure Breathing apparatus.
‡ (v), Value obtained on venous blood.
§ Calculated from venous pH.

changes suggestive of myocarditis were not observed.

2. Blood gas analysis

Arterial blood gas studies were performed on two patients with primary influenza virus pneumonia and six patients with the syndrome believed to represent combined bacterial and virus pneumonia. The results of these determinations are recorded in Table IX.

Six patients were studied early in the course of their illness. All six showed striking reduction in oxyhemoglobin saturation with values ranging from 46.2 to 84.8 per cent. The two patients with the syndrome of influenza virus pneumonia had elevated partial pressures of carbon dioxide and lowered arterial pH. The four patients with mixed viral and bacterial pneumonias had normal or reduced partial pressures of carbon dioxide and serum bicarbonate concentrations early in the course of the disease, with variable alterations in arterial pH.

As their pulmonary disease progressed, the arterial blood gas studies performed on four patients showed remarkably similar abnormalities. Arterial oxyhemoglobin saturation fell progressively, and values of 50.5 and 69.9 per cent were noted in two patients. The remaining two patients were not removed from oxygen for blood gas studies because of profound air hunger and cyanosis. All four patients developed respiratory acidosis as evidenced by a high partial pressure of carbon dioxide and a markedly reduced arterial pH.

Arterial blood specimens obtained from four patients early in the recovery phase showed a return of the oxyhemoglobin saturation towards normal. Three of these four demonstrated continuing carbon dioxide retention during this period of improvement.

3. Studies on renal function

Although urine abnormalities were noted in the majority of patients studied, there was no evidence of direct renal damage accompanying influenza. Proteinuria was present in virtually all patients and appeared to be related to the height of the fever. Hematuria was not noted during the course of illness. Admission blood urea nitrogen values ranged from 11 to 20 mg. per cent. In patients with secondary bacterial pneumonia, blood urea nitrogen concentrations promptly returned to normal levels with hydration. In patients critically ill with diffuse pulmonary syndromes, blood urea nitrogen frequently rose as the disease progressed. Urea clearances performed in a number of patients were within normal limits unless shock had supervened. Inulin clearances performed on three patients critically ill with advanced influenza virus pneumonia were significantly depressed.

4. Studies of serum electrolytes

No consistent changes in serum electrolytes were noted. In general, serum sodium, potassium and chloride determinations were normal. In patients with diffuse pulmonary disease, venous carbon dioxide combining powers frequently rose as the severity of the illness increased, and serum chlorides fell concomitantly. Two patients with primary influenza virus pneumonia and severe acidosis had elevated serum pyruvate levels of 130 and 164 μEq. per L. and elevated blood ketone values of 103 and 142 μEq. per L., respectively.

5. Liver function studies

Liver function studies were performed in nine patients with primary influenza virus pneumonia or mixed influenza virus and bacterial pneumonia. With the exception of alterations in the serum glutamic oxaloacetic transaminase levels, no significant abnormalities were noted.

Serum glutamic oxaloacetic transaminase. Twenty-two patients had single or repeated determinations of serum transaminase concentrations. The results of determinations are graphed in Figure 7. As noted, transaminase levels were elevated in the majority of patients studied. In general, the height of serum transaminase concentrations appeared to parallel the severity of the clinical illness. Patients with secondary bacterial pneumonia generally showed the least abnormality. Two patients with primary influenza pneumonia and seven patients with mixed viral and bacterial pneumonia showed elevations of serum transaminase to over 60 units per ml. per minute at some time during the course of their illness.

6. Studies on blood clotting

Bloody sputum was a prominent feature of primary influenza virus pneumonia and the concom-

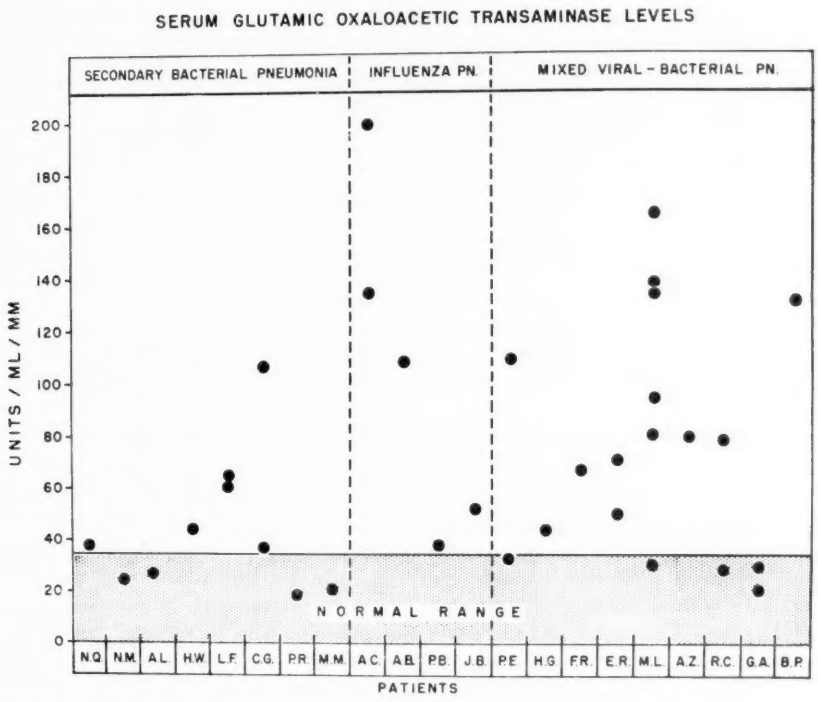

FIG. 7. SERUM GLUTAMIC OXALOACETIC TRANSAMINASE LEVELS IN PATIENTS WITH PULMONARY COMPLICATIONS OF INFLUENZA

itant influenza virus-bacterial pneumonia. Studies of bleeding, clotting and prothrombin times, as well as tourniquet and clot retraction tests and quantitative platelet counts were done in five patients with hemoptysis accompanying their illness. All studies were within normal limits.

7. Other serologic studies

Serum cold agglutinins were determined on one to six occasions in 18 patients. Seventeen patients showed no detectable cold agglutinins. An initial cold agglutinin titer of 1 : 5 in the serum of one was not present in a second serum. Heterophile antibody concentrations in nine patients were within normal limits.

8. Studies on staphylococci isolated from patients with pulmonary complications

Coagulase positive hemolytic *Staphylococcus aureus* strains were present in the admission sputum cultures in 12 patients with bacterial or mixed viral and bacterial pneumonias. In six the staphylococcus was grown in pure culture from the sputum. In the remaining six patients, other potential pathogens were also recovered in significant numbers.

Bacteriophage typing was performed on 10 of the 12 initial sputum isolates. No single phage type predominated. Three staphylococcal strains were typed in bacteriophage group I, three strains in group III, and two strains had miscellaneous bacteriophage patterns. Two staphylococcus strains were nontypable.

Of these initial isolates, five strains were sensitive to less than one unit of penicillin and seven were penicillin-resistant (1.56 to 50 units per ml.). Seven of the 12 initial isolates were sensitive to tetracycline, 10 to streptomycin, 11 to erythromycin, and 11 to novobiocin. All of the strains which were sensitive to penicillin were also susceptible to all other drugs. Data concerning staphylococcal isolates are summarized in Table X.

As noted in Tables X and XI, additional strains

TABLE X

Studies on coagulase positive staphylococcus aureus strains isolated from 12 patients

Patient	Group	Source culture	Hospital day obtained	Amt. growth*	Bacteriophage type	Antibiotic sensitivity†					
						Pen.	SM	EM	CM	T	Nov.
N. Q.	Secondary bacterial pneumonia	Sputum	1	+++		R‡	S‡	S	S	R	S
B. N.		Sputum	1	+	Gr. III 47/75	R	R	SS	SS	R	S 0.39
C. G.		Sputum	1	+++	Gr. I 52A/79	R 12.5	S 1.56	S 0.1	SS 6.25	S 0.4	S 0.39
P. R.		Sputum	1	++	Non-typable	S	S	S	S	S	S
H. W.		Sputum	1	+++	Gr. III 53/VA4	R 6.25	R 50	R 50	R 50	R 50	S
		Sputum	18	++	Gr. III 55/VA4	R 15.6	R 50	R 50	R 50	R 50	S
L. F.		Sputum	1	+++	Miscellaneous 80/81	R 50	S 1.56	S 0.1	SS 6.25	R 50	S 1.56
		Stool	16	+++	Gr. III 53/VA4	R 50	R 50	R 25	R 50	R 50	S 0.39
H. G.	Mixed viral-bacterial pneumonia	Sputum	1	+++		S	S	S	S	S	S
P. E.		Sputum	1	++	Gr. I 52A/79	S 0.05	S	S	S	S	S 0.78
		Naso Pharynx	1	+++							
G. A.		Sputum	1	++	Nontypable	S 0.1	S 1.56	S 0.1	SS 6.25	S 0.4	S 0.78
R. C.		Sputum	1	++	Gr. III 7/47	R 6.25	S	S 0.2	SS 6.25	S 0.8	S 0.39
		Sputum	47	+	Nontypable	R 1.56	R 50	R 50	R	R	S 0.39
A. Z.		Sputum	1	+++	Miscellaneous 80/81	R 25	S 1.56	S 0.1	SS 6.25	R 7.50	R 12.5
		Lung	7	+++ 5×10^8/ml.	Gr. III 53/VA4	R 50	R 50	R 50	R 50	R 50	S 0.39
B. P.		Sputum	1	+++	Gr. I 52A/79	S	S	S	S	S	S
		Lung	2	+++ 4×10^8/ml.		S 0.78	S 0.78	S 0.2	S	S	S 0.39

* +, Occasional colony; ++, appreciable growth; +++, predominating organism and/or pure culture.

† Pen, Penicillin; SM, Streptomycin; EM, Erythromycin; CM, Chloramphenicol; T. Tetracycline; Nov, Novobiocin.

‡ R, resistant; S, sensitive. Where tube dilution sensitivity studies were performed these are recorded in units per ml. for penicillin and μg. per ml. for the other antibiotics.

of staphylococci were isolated from five of these patients 16 to 50 days after the initial isolation of staphylococci. In two patients (H. W. and B. P.), strains possessing the same phage pattern and antimicrobial sensitivities were isolated from the sputum and lung, respectively. In the remaining three patients (L. F., R. C. and A. Z.), strains of different phage types were recovered from stool, sputum and lung. In each instance in which a different staphylococcal strain was isolated during hospitalization, it was found to be resistant to the antimicrobial drugs in use (Table XI). This apparent superinfection within the hospital appeared to be of possible importance in the fatal outcome in one patient (A. Z.).

9. Comparison of the virologic findings in patients with different clinical syndromes

Separation of the clinical syndromes described was made on the basis of clinical evidence before virologic information became available. Differences among clinical syndromes with respect to

TABLE XI

Summary: Antibiotic sensitivities of 17 strains of coagulase positive staphylococic isoalted from patients with influenza and pneumonia

	Penicillin		Streptomycin		Erythromycin		Chloramphenicol		Tetracycline		Novobiocin	
	S*	R*	S	R	S	R	S	R	S	R	S	R
Primary sputum isolations (12 patients)	5	7	10	2	11	1	10	2	7	5	11	1
Second isolations of staphylococci† (5 patients)	1	4	1	4	1	4	1	4	1	4	5	0

* S, sensitive; R, resistant.
† Different phage type in three of five. Source of second isolates: sputum (eighteenth and forty-seventh days of hospitalization), two; lung (second and seventh days of hospitalization), two; stool (sixteenth day of hospitalization), one.

the incidence of virus recovery from throat washings and the concentrations of virus obtained from lungs at autopsy were subsequently noted. These differences are summarized in Table XII.

Throat washings taken from 13 patients with secondary bacterial pneumonia did not yield influenza virus in any patient. In contrast, influenza virus was recovered from throat washings of nine of 12 patients believed to have primary influenza virus pneumonia or mixed viral-bacterial pneumonia. Significant differences were also noted in the concentration of influenza virus in lung tissues obtained from patients dying with primary influenza pneumonia or mixed viral-bacterial infections. Three of the four patients who died with the syndrome of primary influenza virus pneumonia had pulmonary concentrations of influenza virus above 10^4 infectious units (EID_{50}) per Gm. of tissue. In contrast, virus concentrations of less than 10^2 infectious units per Gm. were obtained in lungs of the three of four patients dying with mixed viral-bacterial pneumonia in whom influenza virus could be recovered.

10. Evidence of central nervous system involvement

Three patients showed during their illness abnormalities which suggested the possibility of

TABLE XII

Summary: Influenza virus isolations from throat washings and lung specimens

Secondary bacterial pneumonia				Primary influenza virus pneumonia				Mixed influenza virus-bacterial pneumonia			
Patient	Day of illness specimen obtained	Specimen	Result	Patient	Day of illness specimen obtained	Specimen	Result (conc./Gm. lung)†	Patient	Day of illness specimen obtained	Specimen	Result (conc./Gm lung)†
R. S.	3	TW*	0	G. H.	5	Lung	+++	H. G.	7	TW	0
B. N.	15	TW	0				$10^{5.3}$	P. E.	3	TW	+
C. G.	12	TW	0	C. P.	3	Lung	+++	E. R.	1	TW	+
D. P.	7	TW	0				$10^{5.7}$	G. A.	3	TW	+
M. M.	6	TW	0	A. B.	3	TW	+	R. C.	5	TW	0
R. L.	7	TW	0		4	Lung	+++		53	Lung	0
P. R.	4	TW	0				10^4	M. L.	3	TW	+
N. M.	6	TW	0	P. B.	2	TW	0		? 1–4	TW	+
A. L.	1	TW	0	J. B.	3	TW	+	F. R.	? 6–10	Lung	+
G. M.	13	TW	0		11	Lung	0				10^2
H. W.	35	TW	0		3	TW	+		4	TW	+
L. F.	9	TW	0	A. C.	4	Lung	+	A. Z.	10	Lung	+
A. M.	7	TW	0				10^2				10^2
									4	TW	+
								B. P.	4	Lung	+
											$10^{2.3}$

* TW, throat washing.
† Concentrations of virus recorded in 50 per cent egg infectious doses (EID_{50}).

central nervous system involvement. One patient (J. B.), a 75 year old woman with primary influenza virus pneumonia, entered the hospital in semicoma and remained comatose throughout her 10 day hospital course. Focal neurologic signs were not present. Cerebrospinal pressure, cell count, protein, sugar and chloride were normal on two occasions, and influenza virus was not recovered from cerebrospinal fluid.

A second patient (A. C.) with primary influenza virus pneumonia had a series of convulsions followed by coma 24 hours after admission and expired eight hours later. At autopsy no pathologic abnormalities were detected in the brain.

The third patient (P. E.) with combined bacterial and viral pneumonia initially responded well to oxygen and antimicrobial therapy. On his third hospital day he became lethargic. Twenty-four hours later he was noted to have a partial left III cranial nerve paresis and a mild right hemiplegia. Cerebrospinal fluid was slightly xanthochromic. Cerebrospinal fluid protein was 54 mg. per cent and the cell count was normal. X-rays of the skull were normal and parietal burr holes did not reveal evidence of a subdural hematoma. Electroencephalograms were diffusely abnormal and were felt to be compatible with encephalitis. No virus could be recovered from the cerebrospinal fluid. During the next three weeks the patient remained lethargic, confused, and disoriented. His mentation changes and neurologic signs gradually improved, although at the time of discharge 40 days after admission his electroencephalogram remained markedly abnormal.

Whether the changes noted in these patients were related to severe hypoxia and profound illness or were a direct result of influenza virus infection was not satisfactorily answered by the present study.

III. The course and therapy of influenza with pulmonary complications

1. Influenza with secondary bacterial pneumonia

Patients with secondary bacterial pneumonia following influenza responded to treatment in a manner which did not differ from that observed in bacterial pneumonia uncomplicated by influenza. The eight patients with pneumococcal pneumonia responded promptly to antimicrobial therapy which consisted of penicillin alone in five patients, penicillin in combination with erythromycin and streptomycin in two, and erythromycin and tetracycline in one patient who had a history of penicillin allergy. Temperatures returned to normal in two to eight days and physical findings of pneumonia cleared in four to 16 days.

The five patients with staphylococcal pneumonia following influenza also responded satisfactorily to antimicrobial therapy. Four of these patients had infections produced by strains which were resistant to penicillin but sensitive to erythromycin and chloramphenicol. These four patients received antimicrobial regimens which included erythromycin. The fifth patient, with pneumonia caused by a staphylococcal strain resistant to high concentrations of streptomycin, chloramphenicol and erythromycin, appeared to respond to therapy which utilized all three of these agents. In this group, temperature response was slower, taking from four to 13 days for complete return to the afebrile state. Roentgenograms cleared more slowly. One patient developed roentgenographic evidence of pulmonary cavitation with subsequent slow resolution. The single patient with pneumonia apparently due to *Hemophilus influenzae* responded promptly to therapy with chloramphenicol and erythromycin.

One patient (B. N.) developed her pneumonia while in the hospital. Sputum cultures consistently yielded *Aerobacter aerogenes, Candida albicans* and small numbers of hemolytic *Staphylococcus aureus*. Despite the administration of penicillin, streptomycin, tetracycline, erythromycin and chloramphenicol in varying combinations, she showed no significant improvement. On the thirty-seventh day of her illness the patient died suddenly with symptoms suggestive of a massive pulmonary embolus. No autopsy was obtained on this patient.

2. Primary influenza virus pneumonia

Treatment of the syndrome of primary influenza pneumonia was unsatisfactory, and only one of six patients in this group survived. Major therapeutic problems were the high, unremitting fever, progressive respiratory disease with increasing hypoxia and the increasing respiratory and metabolic acidosis.

TABLE XIII

Comparison of oxygen administration via positive pressure mask or intermittent positive pressure breathing (IPPB) mask on the arterial oxyhemoglobin saturation in patients with influenza virus pneumonia

Patient	Arterial oxyhemoglobin saturation (*per cent*)*		
	Without O_2	Positive pressure mask—100% O_2	IPPB 100% O_2
J. B.	46.2	91.0	96.5
	73.5		91.5
	69.9		94.7†
F. R.	50.5		91.5
A. Z.	71.1	82.6	93.9

* All specimens were taken serially from the brachial artery; 15 to 20 minutes was allowed for stabilization under each test condition before samples were obtained.
† Forty per cent O_2 administered.

Despite the lack of evidence of bacterial infection, each patient received intensive antimicrobial therapy, the details of which are outlined in the individual case reports. In no instance was there any apparent improvement following administration of antibacterial agents.

The severe hypoxia and marked cyanosis were not corrected by the use of an oxygen tent or routine oxygen face mask. Arterial blood oxygen saturation studies performed on three patients indicated that oxygen administered via the Bennett Intermittent Positive Pressure Breathing (IPPB)[4] Apparatus resulted in maximal improvement in oxyhemoglobin saturation. As noted in Table XIII, significant increase in arterial oxygen saturation followed the use of a positive pressure mask, but in each case further improvement in oxygen saturation was achieved by use of the IPPB mask.

Unfortunately, the IPPB mask was not tolerated by most patients for prolonged periods of time. The cycling apparatus proved fatiguing to the patient, and claustrophobia and agitation were commonly intensified by the mask. Also, the introduction of air under pressure was frequently irritating and appeared to dry the pharynx and tracheobronchial passages. This often resulted in thick tenacious secretions which were difficult to remove despite maintenance of high humidity in the mask by alternating aerosols of bronchodilators, Alevaire® and normal saline. The one patient who survived was the only patient able to tolerate intermittent positive pressure breathing for long periods of time.

Five of the six patients, including the patient who survived, received adrenal steroids in doses which varied from the equivalent of 400 mg. to 1,000 mg. of cortisone acetate daily. Steroids used were hydrocortisone, which was administered intravenously, or prednisone, which was given intramuscularly or orally. In the majority of patients the administration of steroids appeared to reduce the fever. In certain instances, discussed in more detail in the case reports, the administration of intravenous hydrocortisone appeared to result in a transient improvement in the pulmonary signs and respiratory distress. Nevertheless, steroids did not appear to alter significantly the overall course of the illness.

3. Coincident influenza virus and bacterial pneumonia

Five of the nine patients with combined viral and bacterial pneumonia recovered despite the critical nature of their illness upon admission. Therapy was similar to that utilized in primary influenza virus pneumonia except for the choice of antibiotics. In each instance, antibacterial therapy with penicillin, streptomycin and either erythromycin or chloramphenicol was administered until a specific bacteriologic agent was isolated and its *in vitro* sensitivity to antibiotics determined. Thereupon the antibiotic regimen was altered when necessary to include the most appropriate antimicrobials. Oxygen therapy was administered in the same manner as in primary influenza virus pneumonia and was fraught with the same difficulties.

Seven of the nine patients also received steroid hormones in dosage equivalent to 400 to 1,000 mg. cortisone acetate daily. Temperatures fell towards normal concurrently with steroid therapy in four of the seven. Dramatic temporary improvement with subsequent relapse was noted following steroid treatment in one patient (R. C.). In the remaining patients there were no striking effects on the clinical course.

Four patients with evidence of combined viral

[4] The IPPB mask used in these studies was obtained from V. Ray Bennett and Assoc., Inc., Los Angeles, Calif.

and bacterial pneumonia died during the course of their illness. One patient (F. R.) with pneumococcal and viral pneumonia died on the sixth day of his illness with a progressive diffuse pulmonary disease in every way similar to that seen in primary influenza virus pneumonia. Lung specimens obtained post mortem were sterile on bacteriologic culture, and influenza virus was recovered in a concentration of 10^2 infectious units (EID_{50}) per Gm.

Two patients (A. Z. and B. P.) died of overwhelming staphylococcal and influenza virus pneumonia. One patient (R. C.) died 53 days after the onset of her initial influenza symptoms with an acute myocarditis and pulmonary arteritis of unknown etiology. Patients A. Z., B. P. and R. C. are described in detail in the case report section.

4. Treatment of the hypercapnea and respiratory acidosis observed in patients with diffuse pulmonary involvement

Seven of the 15 patients with primary viral or combined viral and bacterial pneumonia developed progressive respiratory acidosis with increasing partial pressures of carbon dioxide and falling arterial blood pH. In five patients serum bicarbonate levels rose progressively as carbon dioxide tension rose, resulting in a partially compensated acidosis.

Because of the severe carbon dioxide retention, acetazoleamide (Diamox®), 250 mg., was administered every six hours to four of these patients for periods of several days. The results of this therapy are presented in Table XIV.

Three patients had a diuresis and serum carbon dioxide combining power returned to normal. No significant fall in blood pH occurred during acetazoleamide therapy, and all three patients showed definite clinical improvement with reduction in pulmonary signs and subjective respiratory distress. In the remaining patient (A. Z.) a return of blood carbon dioxide and pH to normal was noted, but no clinical improvement was observed.

IV. Pathologic findings at postmortem examination

1. Primary influenza virus pneumonia

Complete postmortem examinations were performed on four patients who died with the syn-

TABLE XIV

Effects of acetazoleamide on the carbon dioxide retention and respiratory acidosis noted in four patients with influenza pneumonia

Time of study	P. B.				A. Z.				G. A.			P. E.			
	Day of illness	Art. pH units	Art. pCO_2	Art. HCO_3	Day of illness	Art. pH units	Art. pCO_2	Art. HCO_3	Day of illness	Venous pH units	Venous CO_2	Day of illness	Art. pH units	Art. pCO_2	Art. HCO_3	Venous CO_2
		M	*mM Hg*	*mEq./L.*			*mM Hg*	*mEq./L.*			*mM/L.*			*mM Hg*	*mEq./L.*	*mM/L.*
Day after admission	3	7.43	45	28.9	4	7.42	25	16.1	3		22	3	7.46	37	25.7	27
Preceding acetazoleamide	6	7.31	76	37.2	8	7.22	78	30.8	11	7.23	32	16	7.36	52	28.4	36
Post acetazoleamide	8	7.30	47	22.6	9	7.44	39	25.9	13	7.36	23	18				26

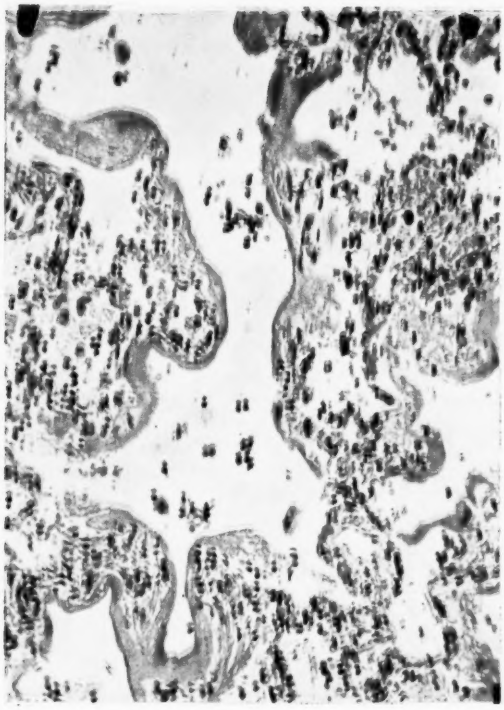

FIG. 8. PRIMARY INFLUENZA VIRUS PNEUMONIA, CASE G. H.

Hyaline acellular membrane lining alveolar ducts and alveoli of the right upper lobe. Note the relative paucity of inflammatory cells or erythrocytes in the alveoli. Hematoxylin and eosin 180 ×.

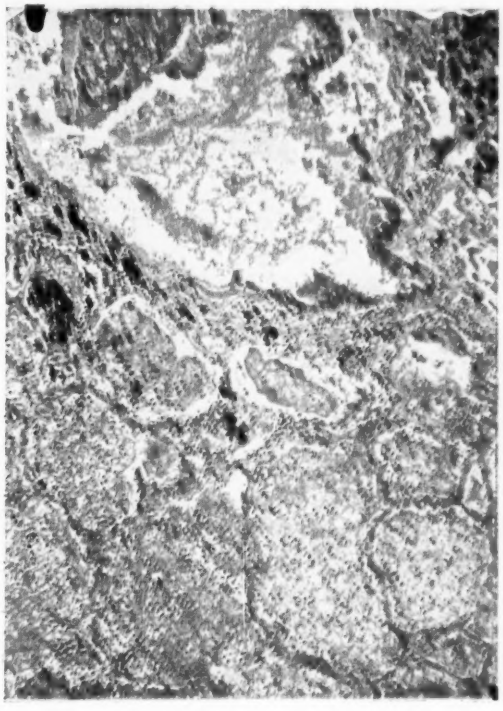

FIG. 9. PRIMARY INFLUENZA VIRUS PNEUMONIA, CASE G. H.

Ulceration of a small bronchus in the right lower lobe. Note that adjacent alveoli are filled with erythrocytes admixed with fibrin. Neutrophilic leukocytes are present, but in small numbers. Hematoxylin and eosin 90 ×.

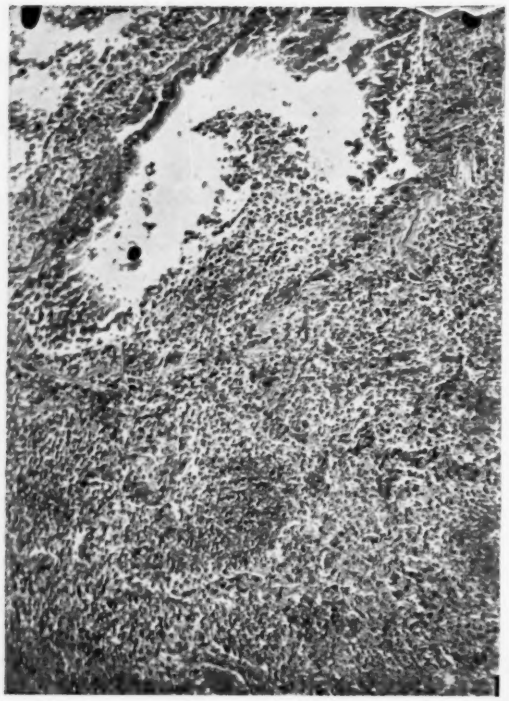

FIG. 10. PRIMARY INFLUENZA VIRUS PNEUMONIA, CASE C. P.

Ulceration of small bronchus in the left lower lobe. Large numbers of neutrophilic leukocytes are present within the lumen and the wall of the bronchus and alveolar spaces. Note the hyperemia of capillaries and alveolar hemorrhage. A few alveolar walls are necrotic. Hematoxylin and eosin 120 ×.

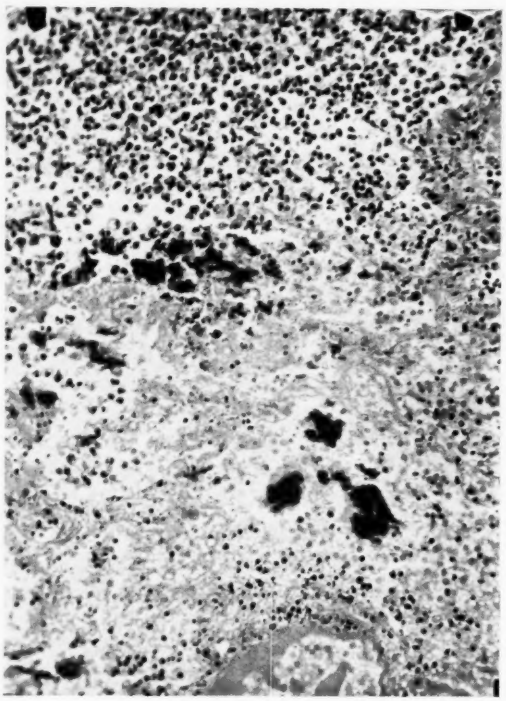

FIG. 11. COMBINED INFLUENZA VIRUS AND STAPHYLOCOCCAL PNEUMONIA, CASE A. Z.

Wall of small abscess showing central zone of necrosis containing colonies of staphylococci and adjacent masses of neutrophilic leukocytes. Hematoxylin and eosin 240 ×.

drome believed to represent influenza virus pneumonia without bacterial involvement. Each of these cases showed distinctive gross and microscopic alterations. High titers of influenza virus were demonstrated in the lungs of three, and pathogenic bacteria could not be demonstrated in culture or in numerous histological preparations of the lungs in these patients (G. H., C. P. and A. C.). The fourth case (J. B.) aspirated preterminally, and a small number of fungi and bacteria were demonstrated about aspirated material within bronchi but did not appear of importance in the causation of the diffuse parenchymal changes. In this case, influenza virus was not recovered from the lung but was isolated from the throat washings during life.

The lungs in each of the four cases presented remarkably similar alterations. The trachea and bronchi often contained bloody fluid, and the mucosa was strikingly hyperemic. The parenchyma was dark red, markedly congested, edematous and heavy. The total weight of both lungs varied from 1,600 to 4,000 Gm. Crepitus was often greatly diminished or absent, especially in the posterior and inferior portions, and these areas were dark purple-red in contrast to the somewhat lighter color of the upper lobes. On section, large amounts of blood-tinged fluid flowed from the dark red surfaces, and irregular hemorrhagic areas, ranging from a few millimeters to 6 cm. across, were frequently encountered. The pulmonary arteries and veins in these areas were devoid of thrombi.

Widespread and striking microscopic alterations were found in varying degrees in all four cases (Figures 8–10). Tracheitis, bronchitis and bronchiolitis with loss of normal ciliated epithelial cells were prominent and were frequently associated with evidence of epithelial regeneration. Submucosal hyperemia, focal hemorrhage, hemorrhage, edema and a slight cellular infiltrate were present in such areas. The alveolar spaces contained varying numbers of neutrophils and mononuclear cells admixed with fibrin and edema fluid (Figures 8–10). The alveolar capillaries were markedly hyperemic and intra-alveolar hemorrhage was common, particularly in the lower lobes (Figure 9). Focal necrosis of the alveolar septa was found in only one case. In general, all changes described were most marked in the lower lobes, as demonstrated in Figures 8 and 9.

In each of the cases, striking acellular, hyaline membranes lined many of the alveolar ducts and alveoli. Such hyaline membranes were frequently prominent in areas that showed minimal cellular exudation.

Examination of the heart revealed chronic cardiac disease in each of the four. Two patients had severe rheumatic valvular damage with severe mitral valve deformities; one patient showed extensive coronary atherosclerosis. Cardiac changes compatible with longstanding hypertension were noted in the remaining patient.

In no instance was there gross or microscopic evidence of myocarditis. Microscopic examination of the brain failed to show evidence of encephalitis in the three cases in which examination was permitted.

2. Combined influenza virus and bacterial pneumonia

Postmortem studies were also performed on four patients believed to have concomitant influenza virus and bacterial pneumonia. Two patients who died on the fifth and tenth days of illness (B. P. and A. Z.) showed gross and microscopic changes generally similar to those described in the primary influenza virus pneumonia group. In addition, both patients had a diffuse, necrotizing pneumonia. Microabscesses in which myriads of gram-positive cocci could be easily demonstrated were found throughout the lungs (Figure 11). Cultures yielded hemolytic *Staphylococcus aureus*. Influenza virus was also recovered from the lungs of both cases. Examination of the heart in both cases failed to show abnormalities. The one brain examined showed no gross or microscopic changes.

In the remaining two patients (R. C. and G. A.), death occurred two months after the onset of influenza symptoms. Influenza virus was not recovered from these lungs post mortem, although the virus had been previously obtained from throat washings.

One patient (R. C.) died 53 days after the onset of influenza. At autopsy, chronic rheumatic heart disease with mitral stenosis was found. In addition, a widespread, acute, necrotizing myocarditis

and subacute pulmonary arteriolitis were present. The lungs failed to show hyaline membrane formation, bronchiolitis or other changes that could be clearly attributed to the initial viral infection. The etiology of the arteriolitis and myocarditis in this patient was not determined. The striking polymorphonuclear infiltration of the myocardium appeared of recent origin.

The remaining patient (G. A., Autopsy No. 17365) with a pneumonia believed to be due to both viral and bacterial infection responded well to therapy and was discharged after complete recovery. He re-entered the hospital six weeks later with a fresh myocardial infarction and subsequently expired. Autopsy studies failed to reveal evidence of residual pulmonary abnormalities that could be attributed to the previous influenza infection. The brain appeared normal. The detailed postmortem findings are included in the individual case reports.

CASE REPORTS

I. Influenza complicated by bacterial pneumonia

The four patients discussed below characterize the spectrum of illness as observed in 15 patients who developed bacterial pneumonia following influenza infection.

1. N. Q., NYH 778883. A 21 year old white man was admitted on Sept. 30, 1957, with fever, chest pain and bloody sputum. Five days prior to admission the patient had noted the onset of a moderately sore throat, chilly sensations, anorexia, photophobia, dry cough and eye ball soreness. On bedrest there was progressive improvement over the next three days. The evening before admission the patient became precipitously worse. He developed pleuritic right anterolateral chest pain, and his cough became productive of bloody sputum.

Physical examination on admission revealed a temperature of 40.5° C., heart rate of 100 beats per minute, respirations of 30 per minute and blood pressure of 150/60 mm. Hg. The patient was lethargic and acutely ill. There was splinting of the right lower thorax on respiration. A right pleural friction rub was present, and dullness to percussion, bronchial breath sounds, egophony and fine inspiratory rales were noted over the right base posteriorly. The remainder of the physical examination was not remarkable.

Initial laboratory studies showed a white blood cell count of 12,200 per cu. mm. with 71 per cent mature polymorphonuclear cells and 10 per cent immature forms. The erythrocyte sedimentation rate was 46 mm. per hour. Sputum smears demonstrated many polymorphonuclear cells and masses of gram-positive cocci. Sputum cultures contained an almost pure growth of hemolytic *Staphylococcus aureus.* This microorganism was resistant to penicillin but sensitive to erythromycin, chloramphenicol, streptomycin and novobiocin. The admission chest roentgenogram, shown in Figure 12, demonstrated a localized right lower lobe infiltrate.

Therapy was initiated with aqueous penicillin, 2.4 million units per day; dihydrostreptomycin, 2 Gm. per day; and erythromycin, 2 Gm. per day. The course of the patient's illness is shown in Figure 13. During the first five days the temperature ranged to above 39° C. and the patient remained extremely ill. A right pleural effusion developed which was sterile upon culture. During the ensuing 18 days the temperature returned gradually to normal, and the pleural effusion slowly resolved. A chest roentgenogram taken on the tenth hospital day showed cavity formation within the area of pneumonia (Figure 14). Complete resolution occurred on continued therapy, and the patient was discharged asymptomatic after 32 days of hospitalization. A serum obtained late in his illness (22 days) showed a complement fixing titer of 1:512 against the Japan 305 strain of influenza virus.

Comment. This patient had rather typical staphylococcal pneumonia following influenza caused by a strain sensitive to most antibiotics. There was a definite period of improvement after influenza, followed by new symptoms consisting of pleural pain, marked exacerbation of cough, and

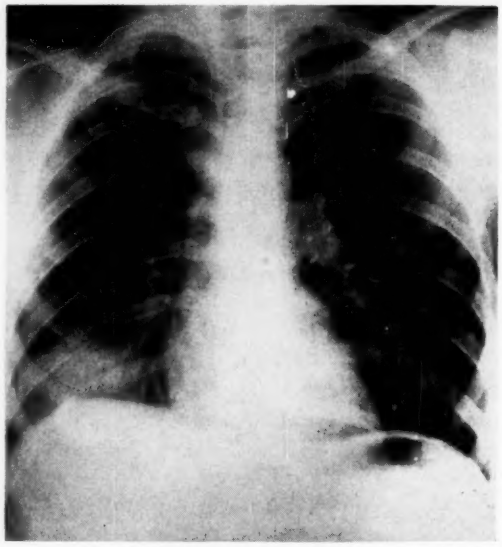

FIG. 12. ADMISSION CHEST ROENTGENOGRAM ON PATIENT N. Q., A 21 YEAR OLD MAN WITH INFLUENZA COMPLICATED BY STAPHYLOCOCCAL PNEUMONIA OF THE RIGHT LOWER LOBE

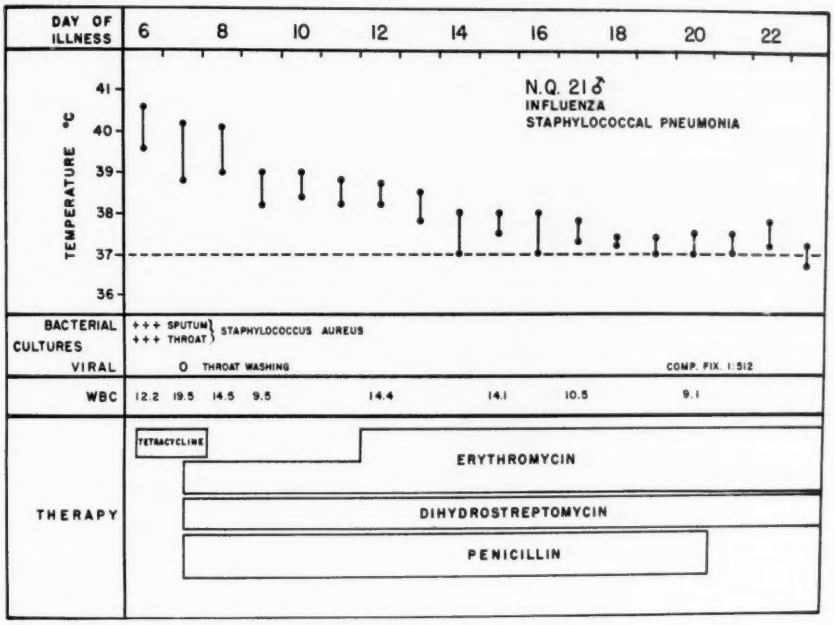

FIG. 13. COURSE OF ILLNESS IN PATIENT N. Q.

bloody sputum which heralded the onset of the staphylococcal infection.

2. C. G., NYH 780147. A 59 year old white man was admitted on Oct. 4, 1957, with fever, chest pain and a productive cough. The patient was well until 11 days prior to admission when he developed fever, headache, sore throat, diffuse myalgias, diarrhea, vomiting and cough which was productive of small amounts of grayish sputum. On bedrest he improved quite markedly so that two days later he was able to return to work. The next day he had a shaking chill and noted the onset of left chest pain, and his cough increased. He was treated with penicillin and tetracycline, but his symptoms continued and he was admitted to the hospital.

Physical examination revealed a well-developed, well-nourished man who appeared in no acute distress. Admission temperature was 38.5° C., pulse 76 beats per minute, respirations 24 per minute, and blood pressure 130/70 mm. Hg. Examination of the chest revealed dullness to percussion posteriorly over the medial part of both lower lung fields. Breath sounds were diminished, and sticky, inspiratory rales were heard over the areas of dullness. The remainder of the physical examination was within normal limits.

The initial white blood cell count was 13,600 leukocytes per cu. mm. with 69 per cent mature polymorphonuclear cells and 17 per cent band forms; the erythrocyte sedimentation rate was 22 mm. per hour. Examination of sputum smears revealed many polymorphonuclear cells and gram-positive cocci. Sputum cultures showed a heavy growth of hemolytic *Staphylococcus aureus* (phage type 52A/79) and *Hemophilus influenzae.* The staphylococcus isolated was resistant to 6.25 units of penicillin and sensitive to streptomycin, erythromycin, tetracycline, chloramphenicol and novobiocin. Chest roentgenogram showed a bilateral lower lobe pneumonia. Influenza virus was not recovered from a throat washing.

The patient was placed on a daily dosage of 2 Gm. each of dihydrostreptomycin, erythromycin and chloramphenicol. He became afebrile by the fourth hospital day and remained so during the remainder of hospitalization. The physical signs of pulmonary involvement cleared slowly. One month after admission, physical and roentgenologic examination were both within normal limits. Serum obtained on Days 14 and 24 showed complement fixing antibody titers of 1:512 and 1:1024 respectively against the Japan 305 strain of influenza virus.

Comment. This patient had a bacterial pneumonia following typical influenza. Both hemolytic *Staphylococcus aureus* and *Hemophilus influenzae* were cultured from the sputum. A distinct period of improvement after influenza was followed by symptoms suggesting bacterial pulmonary infection. Complete recovery followed prolonged antimicrobial therapy.

3. D. P., NYH 495539. A 15 year old school girl was admitted on Oct. 11, 1957, with fever, headache and a

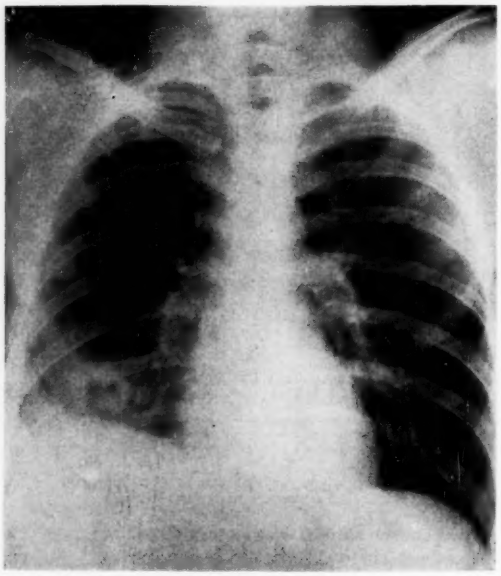

FIG. 14. CHEST ROENTGENOGRAM ON PATIENT N. Q. ON DAY 10 OF HOSPITALIZATION SHOWING A MULTILOCULATED CAVITY IN THE AREA OF PNEUMONIA

productive cough. Five days prior to admission the patient developed a frontal headache which was rapidly followed by anorexia, chilliness, fever and diffuse myalgia. A cough developed which was productive of small amounts of yellow, thick sputum. Three days before admission she had returned to school, but profound malaise, anorexia and productive cough persisted.

At admission, physical examination revealed a temperature of 39° C., pulse rate 100 beats per minute, respirations 28 per minute, and blood pressure of 108/80 mm. Hg. Splinting of the left hemithorax and tenderness of the left lower chest to palpation were noted. Signs of consolidation were evident over the left lower lung field posteriorly, and a pleural friction rub was present. Physical examination was otherwise within normal limits.

The initial white blood count was 10,200 per cu. mm. with 60 per cent polymorphonuclear leukocytes, 17 per cent immature granulocytes, and the erythrocyte sedimentation rate was 34 mm. per hour. Sputum smears showed many gram-positive diplococci, and cultures of the nose, throat and sputum disclosed large numbers of pneumococci, type VIII. Mouse inoculation of sputum from the patient also yielded the same organism. The admission chest roentgenogram revealed a left lower lobe infiltrate.

Therapy was instituted with 300,000 units of procaine penicillin administered every 12 hours. The patient became afebrile 18 hours after admission and remained so during the remainder of her hospitalization. Chest roentgenograms showed complete clearing of the pneumonic infiltrate by the eighth hospital day. A 16-fold rise in complement fixing antibody to influenza A virus (Japan 305 strain) was demonstrated with paired sera obtained on the sixth and sixteenth day of the patient's illness.

Comment. This patient developed pneumococcal lobar pneumonia following typical influenza. In this instance the bacterial complication blended with the initial viral infection. At no time during her illness was there a definite change in symptomatology which dated the onset of her bacterial infection. The response on appropriate antibiotic therapy was satisfactory.

4. R. L., NYH 506121. A 22 year old white man with severe muscular dystrophy was admitted on Oct. 17, 1957, because of fever, dyspnea and chest pain. Six days prior to admission he developed an illness characterized by diffuse myalgia, fever, cough and bifrontal headache. Over the next three days he improved but then noted a sudden exacerbation of symptoms with increased cough productive of blood-streaked sputum, chest pain and dyspnea. These symptoms became progressively worse and necessitated hospitalization.

On admission, physical examination revealed a young man with extensive muscular dystrophy who was in moderate respiratory distress. There was minimal cyanosis of the lips and nailbeds. The admission temperature was 39.6° C., heart rate 110 per minute, respirations 22 per minute, and blood pressure 120/80 mm. Hg. Bilateral inspiratory sticky rales were heard over the lower lobes posteriorly, and signs of consolidation were evident over the right upper lobe. The remainder of the physical examination was unremarkable.

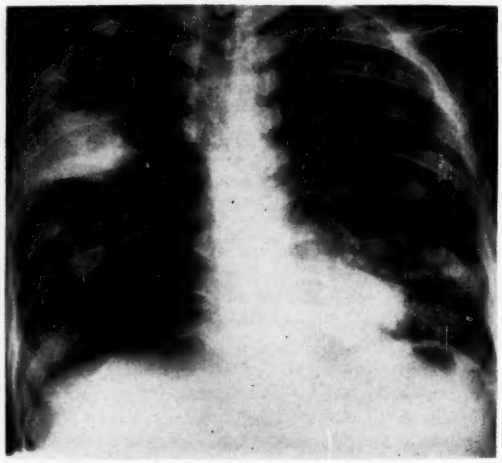

FIG. 15. ADMISSION CHEST ROENTGENOGRAM ON PATIENT R. L., A 32 YEAR OLD MAN WITH INFLUENZA COMPLICATED BY PNEUMOCOCCAL PNEUMONIA OF RIGHT UPPER, RIGHT LOWER AND LEFT LOWER LOBES

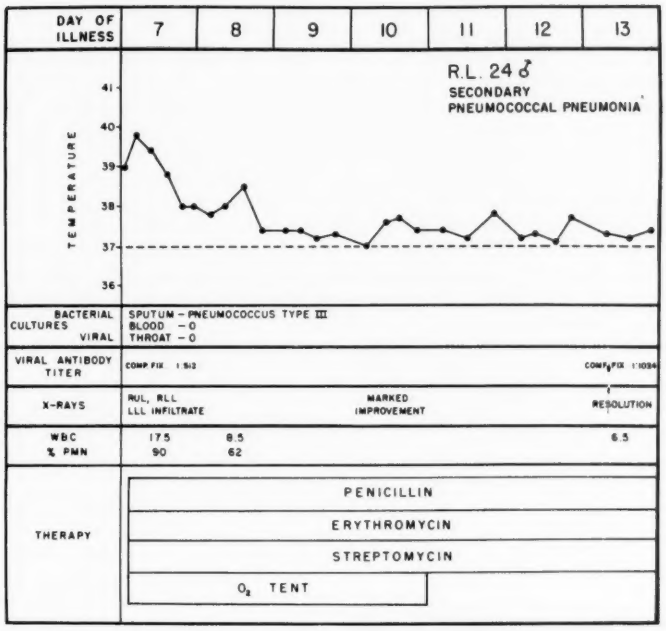

FIG. 16. COURSE OF ILLNESS IN PATIENT R. L.

The admission white blood cell count was 17,500 cells per cu. mm. with 90 per cent polymorphonuclear leukocytes. The carbon dioxide combining power was 32 mM per L. A chest roentgenogram revealed infiltrates of the right upper lobe, right lower lobe and left lower lobe (Figure 15). No sputum could be raised by the patient upon admission. Large numbers of pneumococci, type III, were demonstrated in admission throat cultures. Blood cultures were sterile.

Influenza virus was not isolated from initial throat washings obtained on Day 7 of illness, but complement fixing antibody titers of 1:512 and 1:1024 were demonstrated with serums taken on the seventh and eighteenth days of illness.

The patient's course is pictured in Figure 16. Therapy consisted of oxygen by tent; streptomycin, 2 Gm. per day; erythromycin, 2 Gm. per day; and penicillin, 2.4 million units per day. The patient became afebrile in 48 hours and recovery thereafter was uneventful.

Comment. This patient with muscular dystrophy had typical influenza followed three days later by pneumococcal pneumonia involving three lobes. Prompt and uneventful recovery followed institution of antimicrobial therapy.

II. Primary influenza virus pneumonia

Six patients in the present series had influenza virus infection without demonstrable bacterial infection. Because of the importance of documenting this syndrome, the essential data relative to all six patients are reported here.

1. G. H., NYH 746483. A 49 year old man with long standing rheumatic mitral stenosis and insufficiency was admitted on Oct. 5, 1957, with a 20 hour history of fever and progressive respiratory difficulty. The day prior to admission the patient had developed generalized myalgia, headache, fever and a nonproductive cough. During the next 18 hours his cough became progressively worse and productive of bloody sputum. Dyspnea increased relentlessly and precipitated his admission.

On admission the patient appeared cyanotic, markedly dyspneic and critically ill. The admission temperature was 40° C., the pulse was irregular at 90 beats per minute, respirations were 40 per minute, and blood pressure 125/80 mm. Hg.

The neck and arm veins were not distended. Examination of the chest revealed no evidence of local consolidation. Coarse inspiratory rales and occasional rhonchi at both lung bases posteriorly were the only pulmonary findings, despite the severe respiratory distress. The heart was markedly enlarged and had a mitral configuration. The rhythm was that of auricular fibrillation. A harsh, blowing systolic murmur was heard at the apical area, but because of the patient's stertorous breathing no other murmurs could be distinguished. The liver was not enlarged and peripheral edema was not detected.

Laboratory studies revealed a hemoglobin concentra-

tion of 18 Gm. and a white blood cell count of 19,200 cells per cu. mm. with 24 per cent polymorphonuclear cells, 64 per cent band forms and 6 per cent metamyelocytes. Venous pressure was 90 mm. saline at the level of the right auricle. An electrocardiogram did not differ from previous tracings. Blood urea nitrogen was 20 mg. per cent, and the venous carbon dioxide combining power 22 mM per L. A sputum smear revealed moderate numbers of leukocytes and rare gram-positive cocci. Only a rare colony of pneumococci plus normal pharyngeal flora were grown on culture. The initial chest roentgenogram, shown in Figure 17, revealed an extensive, bilateral, diffusely nodular infiltrate fanning peripherally from the hilar regions. A fusiform shadow seen in the right mid-lung field was believed to represent an interlobar effusion in the right horizontal fissure. This had been present in roentgenograms taken prior to the present illness.

The patient's course is depicted in Figure 18. He was placed in an oxygen tent, and treatment with penicillin, streptomycin and chloramphenicol was inititated. The administration of morphine sulfate, digitoxin, diuretics, aminophyllin and Vaponephrine® by nebulizer did not notably improve his respiratory distress. The patient's temperature rose to 40.9° C. and pulmonary rales increased bilaterally. During the second 24 hours he showed transient improvement, but this was followed by steady deterioration. Inspiratory rales were gradually replaced by expiratory rales, low-pitched grating rhonchi and coarse breath sounds of diminished intensity. Cyanosis was progressive and dyspnea became unbearable. The patient became extremely agitated and was unable to tolerate an oxygen mask of any sort except for two to three minutes at a time. Seventy-two hours after admission his blood pressure became unobtainable. Despite intensive therapy, which included hydrocortisone and levarterenol, the patient died on the fourth hospital day.

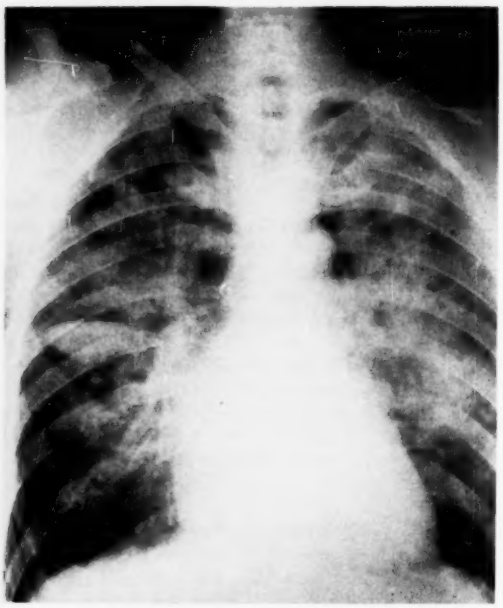

FIG. 17. ADMISSION CHEST ROENTGENOGRAM ON PATIENT G. H., A 49 YEAR OLD MAN WITH PRIMARY INFLUENZA PNEUMONIA SUPERIMPOSED UPON RHEUMATIC HEART DISEASE WITH MITRAL STENOSIS AND INSUFFICIENCY

The density in the right mid-lung field represents a loculated interlobar effusion which was present prior to the acute illness.

Findings at autopsy (Autopsy No. 17247). The significant postmortem findings were confined to the lungs and heart. The heart weighed 600 Gm. and showed changes characteristic of rheumatic heart disease with severe mitral stenosis. The mitral valve was thickened and stenotic. The aortic valve cusps were rigid and thickened. The chambers of the heart were dilated and the right and left ventricles were hypertrophied. The left auricular appendage contained a well-organized mural thrombus. Multiple sections of the heart examined microscopically showed no evidence of myocarditis.

The lungs were intensely engorged and failed to collapse when the chest was opened. Their combined weight was 4,000 Gm. The trachea and bronchi contained much foamy, blood-stained fluid, and the underlying mucosa was markedly hyperemic and exhibited many focal ulcerations. The parenchyma throughout all lobes was dark red and subcrepitant, and large amounts of thin, blood-tinged fluid flowed from the cut surface.

On microscopic examination the mucosa of the trachea and major bronchi was focally ulcerated or covered only by a low cuboidal epithelium. The submucosa in such areas showed marked vascular congestion, focal hemorrhages, edema and a diffuse but scanty infiltration with lymphocytes, plasma cells and neutrophils. The process was patchy, however, and some bronchi were lined by ciliated epithelial cells that appeared essentially normal.

In the parenchyma of the upper lobes the chief alterations consisted of hyperemia of alveolar capillaries, focal alveolar hemorrhages, and a very prominent thick, acellular hyaline membrane that covered the surfaces of the majority of the alveoli and alveolar ducts (see Figure 8). Many alveoli were filled with a pink, granular precipitate admixed with strands of fibrin, a moderate number of erythrocytes and neutrophils, and many histocytes containing hemosiderin or carbon particles. The ciliated epithelium of the small bronchi in the upper lobes appeared essentially normal.

In the lower lobes abnormalities were similar but more marked (see Figure 9). Many small bronchi were ulcerated or covered by a low cuboidal epithelium which exhibited considerable numbers of mitoses. In some areas, many alveoli contained large numbers of neutrophils and hemorrhage was more widespread. Bacteria could not be demonstrated in multiple sections specially stained by the Gram and Giemsa methods.

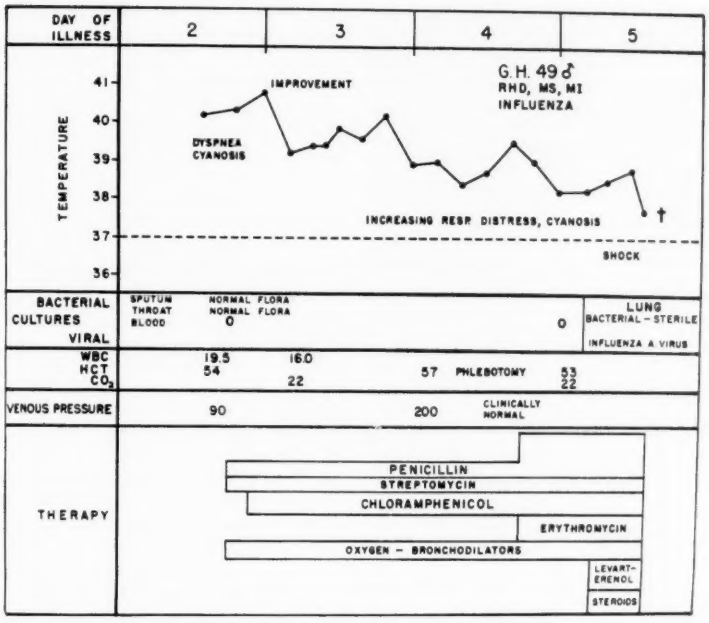

FIG. 18. COURSE OF ILLNESS IN PATIENT G. H.

Bacteriologic cultures performed independently in two laboratories failed to reveal growth of bacteria. Influenza A virus of the Asian type was recovered from lung tissue in concentrations of $10^{5.3}$ infective virus particles per Gm.

Examination of the brain failed to show encephalitis or other unusual changes.

2. C. P., NYH 667901. A 47 year old white man was admitted to the hospital on Oct. 11, 1957, with fever and rapidly progressing dyspnea. The patient had known rheumatic heart disease with predominating mitral insufficiency of many years duration. Chronic exertional dyspnea and orthopnea had been present for at least five years. The day prior to admission the patient developed fever, shaking chills, headache, myalgia and a severe nonproductive cough. During the night his cough became productive of thick, white sputum, and he developed marked respiratory distress for which he was admitted to the hospital.

Physical examination revealed an acutely ill, cyanotic man in severe respiratory distress. The admission temperature was 39.5° C., heart rate 128 beats per minute, respirations 38 per minute, and blood pressure 130/80 mm. Hg. The neck veins were not distended. Percussion of the chest revealed no local areas of dullness. Fine, moist rales were heard throughout the chest accompanied by diffuse inspiratory and expiratory wheezes. Coarse, sticky, inspiratory rales were present at the right base. The heart was grossly enlarged to percussion, and loud apical systolic and diastolic murmurs were noted. There was no peripheral edema.

The white blood cell count obtained on admission was 15,300 cells per cu. mm. with 82 per cent mature polymorphonuclear cells and 18 per cent band forms. The hematocrit was 49 per cent; the erythrocyte sedimentation rate 14 mm. per hour. Urinalysis gave a 4 plus test for protein; the carbon dioxide combining power was 29 mM per L. Venous pressure was 120 mm. saline at the level of the right auricle. An electrocardiogram revealed no changes from previous tracings.

Examination of stained sputum smears failed to reveal any predominating microorganisms. Sputum cultures yielded a sparse, mixed flora in which a few colonies of *Diplococcus pneumoniae, Pseudomonas pyocyaneus,* and *Proteus vulgaris* could be identified. Blood cultures were sterile. The initial chest roentgenogram, shown in Figure 19, demonstrated a greatly enlarged heart and a diffuse, bilateral infiltrate radiating from the hilum which was compatible with either a diffuse pneumonitis or acute pulmonary edema.

The patient was given oxygen by mask, and penicillin, streptomycin and erythromycin were administered. Digitalization was increased and aminophyllin was given intravenously without significant improvement in the pulmonary findings. Because of the patient's profound air hunger, attempts were made to administer oxygen via a positive pressure mask or the IPPB mask, but the patient became markedly agitated and extremely weak with either mask and would not tolerate such therapy. The morning after admission he became rapidly worse, developed increasing respiratory distress and cyanosis, and his blood pressure fell to shock levels. He was placed

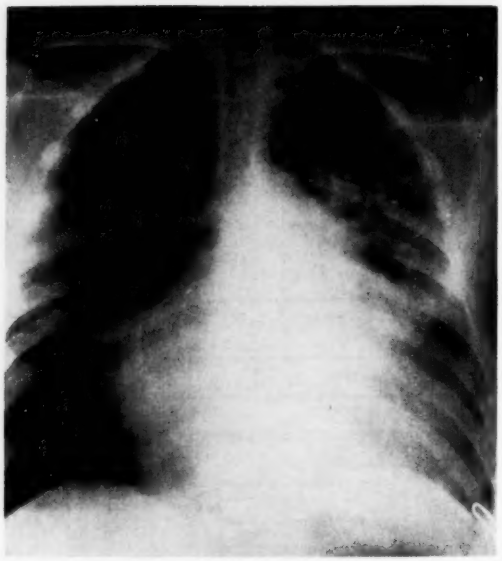

FIG. 19. ADMISSION CHEST ROENTGENOGRAM ON PATIENT C. P., A 47 YEAR OLD MAN WITH PRIMARY INFLUENZA VIRUS PNEUMONIA SUPERIMPOSED UPON RHEUMATIC HEART DISEASE WITH MITRAL INSUFFICIENCY

on levarterenol and was given 500 mg. of hydrocortisone intravenously. The patient became progressively more hypoxic and cyanotic, had a convulsion and died approximately 60 hours after the onset of his illness.

Findings at autopsy (Autopsy No. 17257). The principal findings were confined to the heart and lungs. The heart weighed 650 Gm. and showed changes characteristic of rheumatic heart disease with involvement of the mitral, aortic and tricuspid valves. The mitral valve appeared grossly incompetent because of shortening and fusion of the chordae tendineae and thickening of the leaflets at the line of closure. The aortic valve showed shortening of the left cusp and fusion of the commissures of the posterior and left cusps. The tricuspid valve was thickened along the lines of closure. There was marked hypertrophy and dilatation of both ventricles. The pericardial cavity was obliterated by fibrous adhesions. Microscopic sections of the heart showed a few areas of focal fibrosis but no other unusual changes.

The combined weight of the lungs was 2,100 Gm. and the parenchyma was diffusely firm, noncrepitant, and dark red. The trachea and major bronchi contained much blood-stained mucus, and the bronchus to the left upper lobe was plugged with bloody mucus. The cut section of the lungs revealed numerous dark red nodular areas of hemorrhage. The pulmonary arteries and veins appeared grossly normal in hemorrhagic area and were devoid of thrombi.

On microscopic examination the mucosa of the trachea and larger bronchi was focally denuded or ulcerated. In a few areas only the basal cell layer of the mucosa remained. The blood vessels of the submucosa were markedly hyperemic, and the interstitial tissue was edematous and infiltrated with many lymphocytes and plasma cells and lesser numbers of neutrophils. Many of the smaller bronchi contained plugs composed of mucus and neutrophils.

The alveolar capillaries were engorged with erythrocytes and there were numerous focal areas of alveolar hemorrhage. Many of the alveolar spaces contained a pink granular precipitate and small strands of fibrin. Only a small number of alveolar walls were covered by hyaline membranes. The alveolar spaces of the upper lobes contained considerable numbers of hemosiderin-laden macrophages and only a few neutrophils, while in the lower lobes exudation of neutrophilic leukocytes was more marked, as shown in Figure 10. The mucosa of many of the smaller bronchi was ulcerated and there was necrosis of adjacent alveolar walls and hemorrhage with a moderate interstitial edema. No unusual changes were noted in the brain or other viscera.

Numerous sections of various lobes were stained by Gram's method and bacteria could not be demonstrated. Multiple cultures of lung tissue showed no bacterial growth on prolonged incubation. The Asian strain of influenza A virus was recovered from lung tissue in concentrations of $10^{6.7}$ infectious units per Gm.

3. A. B., NYH 757624. A 45 year old white man with rheumatic mitral insufficiency and aortic stenosis was admitted on Oct. 13, 1957, with fever and rapidly increasing dyspnea. For one year prior to admission the patient had noted exertional dyspnea and mild orthopnea. One and one-half days prior to admission he had developed fever, bifrontal headache, generalized myalgia, a moderate sore throat and a nonproductive cough. On the ensuing day he noted rapidly progressing respiratory distress which caused him to seek admission.

Examination revealed a cyanotic, dyspneic man who was acutely ill. His admission temperature was 40° C., pulse 104 beats per minute, respirations 36 per minute, and blood pressure 120/80 mm. Hg. The pharynx was moderately injected. Venous distention was not present. Examination of the chest revealed no local signs of consolidation. Bilateral sticky, inspiratory rales were present at both bases and harsh breath sounds were present throughout both lung fields. The heart was moderately enlarged, and apical and aortic systolic murmurs were present. No hepatomegaly or peripheral edema were noted. The remainder of the physical examination was within normal limits.

The initial white blood cell count was 13,500 cells per cu. mm. with 78 per cent mature polymorphonuclear cells and 10 per cent band forms. The erythrocyte sedimentation rate was 14 mm. per hour. Urinalysis revealed a 3 plus test for protein. Peripheral venous pressure was 120 mm. saline at the level of the right auricle, and an Evans blue dye blood volume determination was normal. An electrocardiogram taken on the day of admission was considered normal.

Examination of stained sputum smears revealed no predominating microorganisms. Throat and sputum cultures yielded only normal pharyngeal flora with occasional colonies of pneumococci and rare colonies of *Hemophilus influenzae*. Blood cultures were sterile. The admission chest roentgenogram, shown in Figure 20, demonstrated cardiac enlargement which had not changed from previous examinations. There were bilateral diffuse nodular infiltrations compatible with a diffuse pneumonitis or pulmonary edema. A loculated effusion in the right transverse fissure was noted.

The patient was placed in an oxygen tent and penicillin, streptomycin and erythromycin were administered. The patient's condition gradually deteriorated during the first 24 hours. Temperature remained at 40° C. and could not be reduced with large doses of aspirin. Conventional therapy of acute pulmonary edema produced no change in his condition. The respiratory rate rose to 64 per minute and cyanosis deepened. Diffuse inspiratory and expiratory rales increased. Oxygen administered via the IPPB apparatus was tolerated poorly because of agitation and increasing claustrophobia.

At this time hydrocortisone, 100 mg., was given intravenously. The patient improved transiently with marked clearing of the pulmonary rales. However, within six hours his respiratory rate again rose to 64 per minute and cyanosis and agitation increased. Progressive expiratory difficulties were noted and coarse rales, grating rhonchi and harsh breath sounds were heard throughout the lung fields. Venous pressure determined at this time was 65 mm. saline. Again intravenous hydrocortisone was administered, with transient clearing of pulmonary signs. Nevertheless, respirations became increasingly labored and the patient died 40 hours after hospitalization.

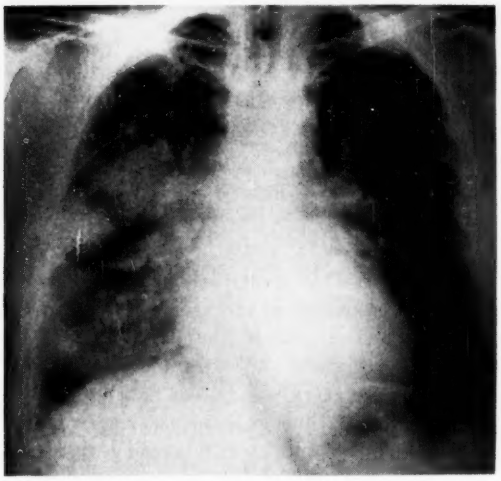

FIG. 20. ADMISSION CHEST ROENTGENOGRAM ON PATIENT A. B., A 45 YEAR OLD MAN WITH INFLUENZA VIRUS PNEUMONIA SUPERIMPOSED UPON RHEUMATIC HEART DISEASE WITH AORTIC STENOSIS AND MITRAL INSUFFICIENCY

The density in the right mid-lung field represents an interlobar effusion.

An autopsy was not performed on this patient. However, needle aspirates of lung were obtained post mortem and were sterile on bacteriologic culture. Influenza A virus of the Asian type was recovered from a throat washing obtained on Day 2 of the illness and also from pulmonary tissue obtained after death in a concentration of 10^4 egg infectious units per Gm.

Comment. Each of these three patients had severe rheumatic heart disease. Each entered the hospital within 36 hours after the onset of a typical influenza-like illness with fever and rapidly progressive difficulties in breathing. In each case, the findings on initial physical examination and chest roentgenograms were interpreted as being compatible with pulmonary edema of cardiac origin. The absence of venous distention, the presence of normal venous pressures, the striking fever and the failure of the patient to respond to treatment for congestive failure suggested that the illness was primarily a manifestation of intrinsic pulmonary disease rather than myocardial decompensation.

Each patient had a rapid down-hill course characterized by sustained fever, increasing cyanosis and air hunger. Marked agitation developed, and measures required to obtain adequate oxygenation could not be tolerated. There was no response to massive antimicrobial therapy and death in all three ensued within 96 hours after hospitalization. At autopsy the lungs showed destructive changes characterized by pulmonary hyperemia and hemorrhages, alveolar hyaline membranes, and focal ulceration of the trachea and bronchi. Postmortem bacterial cultures were uniformly sterile, and influenza A virus (Asian strain) was recovered in high concentration from samples of lung tissue obtained post mortem.

4. P. B., NYH 420164. A 45 year old white man was admitted on Oct. 14, 1957, because of fever and increasing dyspnea. The patient had known rheumatic heart disease of long standing. Six years prior to admission he had undergone mitral valvulotomy and had remained relatively free of cardiac symptoms since that time. The day prior to admission the patient noted the onset of sore throat, fever, a nonproductive cough, diffuse myalgia, nausea and a scratchiness of his eyes. Over the next 12 hours fever persisted. His cough became pro-

ductive of blood-tinged sputum, and he noted increasing difficulty in breathing.

Admission physical examination revealed a dyspneic, cyanotic, agitated man who was acutely ill. The temperature was 40.4° C., heart rate 110 beats per minute, respirations 38 per minute, and blood pressure 150/80 mm. Hg. The conjunctivae were mildly injected. There was no venous distention. Examination of the chest revealed no signs of consolidation, but breath sounds were markedly decreased throughout and a few bilateral inspiratory basilar rales were noted. The heart was enlarged and auricular fibrillation was present. A blowing systolic murmur and a soft mid-diastolic murmur were noted at the apex. There was no hepatomegaly or peripheral edema.

Initial white blood cell count was 13,700 cells per cu. mm. with 71 per cent polymorphonuclear cells and 18 per cent band forms. The hematocrit was 44. Urinalysis showed a 4 plus test for protein. Admission carbon dioxide combining power was 30 mM per L. A venous pressure performed with difficulty on admission was 160 mm. saline, but a blood volume determination simultaneously performed was normal. An electrocardiogram was not significantly different from previous tracings. Arterial oxyhemoglobin saturation was 84.8 per cent and the arterial blood pH was normal.

Sputum smears revealed only sparse bacteria, and sputum cultures yielded normal pharyngeal flora with rare colonies of pneumococci and *Hemophilus influenzae*.

FIG. 21. ADMISSION CHEST ROENTGENOGRAM ON PATIENT P. B., A 45 YEAR OLD MAN WITH PRIMARY INFLUENZA VIRUS PNEUMONIA SUPERIMPOSED UPON RHEUMATIC HEART DISEASE WITH MITRAL STENOSIS

A mitral valvulotomy was performed on the patient six years prior to the present admission at which time the anterior portion of the fourth rib was resected.

Blood cultures were sterile. The admission chest roentgenogram showed patchy pneumonic densities extending from the hila toward both lower lung fields (Figure 21).

The hospital course is illustrated in Figure 22. The patient was given penicillin, 2.4 million units; streptomycin, 2 Gm.; and erythromycin, 2 Gm. daily. In addition, mercurial diuretics and digitalis were administered, and prednisone, 200 mg. daily, was started. Oxygen was administered by tent or nasal catheter alternating with the IPPB mask. Vaponephrine® and Alevaire® were intermittently aerosolized through the mask.

During the first three days of hospitalization the patient appeared to improve. His fever diminished, pulmonary signs lessened and arterial oxygen saturation rose to 91.8 per cent. However, on the fourth day the patient became worse. His temperature rose to 39.2° C., the respiratory rate increased to 44 per minute, and the patient was increasingly disoriented and apprehensive. Examination of the lungs at this time revealed suppression of breath sounds and marked inspiratory and expiratory moist rales, and wheezes were noted throughout the chest. The patient, although extremely restless, tolerated oxygen via the IPPB mask extremely well, and arterial oxygen saturation could be maintained at almost normal levels. Nevertheless, there was evidence of increasing respiratory acidosis.

On the seventh hospital day the arterial pCO_2 had reached 76 mm. Hg. Serum bicarbonate had risen concomitantly to 37.2 mEq. per L. At this time acetazolamide, 0.5 Gm. every 12 hours, was administered. During the next 24 hours the patient had a diuresis of 3,000 ml. of urine. His serum bicarbonate and carbon dioxide combining power fell to a normal range and abnormal pulmonary findings decreased.

During the remaining weeks of hospitalization the patient's inspiratory and expiratory wheezes and rales gradually disappeared and breath sounds became normal. Influenza virus was not demonstrated in a throat washing taken on Day 2 of illness, but convalescent serums showed a 16-fold rise in complement fixing and hemagglutinating antibody titers against the Asian strain of influenza A virus.

Comment. This case represents the only patient who recovered in the group of six patients believed to have primary influenza virus pneumonia. The history, physical findings and initial course were in every way similar to other patients in this group. Although influenza virus was not isolated from the initial throat washing, there was a diagnostic rise in complement fixing and specific hemagglutination-inhibiting antibody titer during the illness. No evidence of concomitant bacterial infection was obtained.

Although the reasons for this patient's survival are not known, two factors may have contributed to his recovery. First, in contrast to the remaining

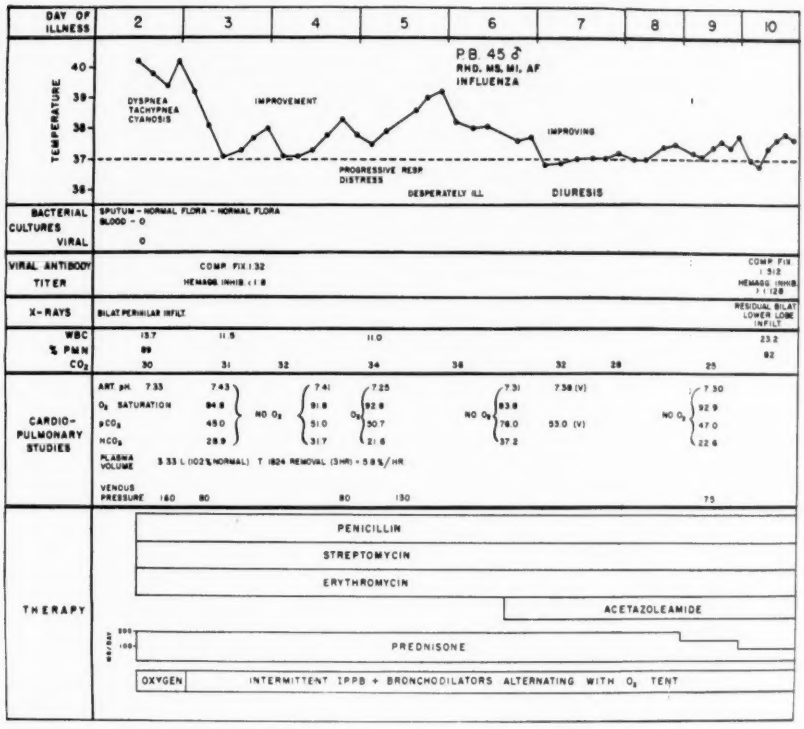

FIG. 22. COURSE OF ILLNESS IN PATIENT P. B.

patients in this group, this patient was able to tolerate oxygen administered via the IPPB mask for long periods and maintained his arterial oxygen saturation at almost normal levels. Secondly, a diuresis occurred in association with the administration of acetazoleamide. Clearing of pulmonary signs and the return of arterial pCO_2 and serum bicarbonate to normal levels were temporally associated.

5. J. B., NYH 779866. A 75 year old white woman was admitted on Oct. 15, 1957, with fever, intense cyanosis and coma. An inadequate history from relatives indicated that one day prior to admission the patient had developed fever which was followed by dyspnea and orthopnea.

Physical examination revealed an intensely cyanotic, comatose, elderly woman with rapid, labored respirations. Admission temperature was 39.2° C., heart rate 108 beats per minute, respirations 54 per minute, and blood pressure 180/80 mm. Hg. Despite the obvious dyspnea, neck veins were not distended. Examination of the chest revealed only diffuse inspiratory rales throughout both lung fields without evidence of local consolidation. The heart was minimally enlarged and a loud, harsh systolic murmur was heard along the left sternal border and at the apex. The liver was not enlarged. There was no peripheral edema.

Initial laboratory studies revealed a white blood cell count of 15,700 cells per cu. mm. with 79 per cent polymorphonuclear cells and 15 per cent band forms. Urinalysis gave a one plus test for protein. Examination of the sediment revealed 8 to 10 white blood cells per high power field. A determination of venous carbon dioxide combining power was 18 mM per L. Peripheral venous pressure was 94 mm. saline. The electrocardiogram showed changes suggestive of left ventricular hypertrophy. A blood volume determination performed on the first hospital day was minimally elevated (118 per cent of calculated normal).

Cultures of sputum, nasopharyngeal and throat secretions yielded only normal pharyngeal flora, and blood cultures were sterile. A throat washing taken on admission revealed the Asian strain of influenza A virus. The admission chest roentgenogram revealed dense pulmonary infiltrations involving the lower half of both lung fields and a prominent hilar fan which was interpreted as vascular congestion.

Because of the similarity of this patient's illness to the cases already described, it was believed that this illness probably represented influenza, and treatment was insti-

tuted accordingly. Digitalization, the administration of morphine sulfate, rotating tourniquets, intravenous aminophyllin, diuretics and positive pressure oxygen via the IPPB apparatus did not produce notable improvement. Penicillin, 6 million units; streptomycin, 2 Gm.; chloramphenicol, 2 Gm.; and hydrocortisone, 200 mg. daily, were given.

The patient's course is depicted in Figure 23. On the program outlined, her temperature returned to normal over a 48 hour period and the respiratory rate decreased to 25 to 30 per minute, but pulmonary findings of rales, rhonchi and expiratory prolongation persisted at the lung bases. Arterial oxygen saturation was difficult to maintain. Serial arterial blood gas studies were performed which are included in Tables IX and XIII and Figure 23. Without supplemental oxygen, arterial oxyhemoglobin saturation was 46.2 per cent on admission and never rose above 73.5 per cent during hospitalization. However, the administration of oxygen under pressure consistently brought oxyhemoglobin saturation to above 90 per cent. There was little evidence that the pCO_2 could be reduced by any mechanical breathing aid. A respiratory acidosis was generally maintained, and superimposed metabolic acidosis gradually developed as the blood urea nitrogen rose. Blood ketones were elevated to 142 μEq. per L., and blood pyruvate was increased to 164 μEq. per L. on the one determination performed.

In view of the continuing coma, lumbar puncture was performed. There were no abnormalities of protein, sugar or cells, and influenza virus could not be recovered from the cerebrospinal fluid. The blood urea nitrogen gradually rose to a maximum of 83 mg. per cent. On the ninth day of disease the patient aspirated food and pharyngeal secretions; respiratory findings increased and coma deepened. She expired on the eleventh day of illness.

Findings at autopsy (Autopsy No. 17278). The principal abnormalities were confined to the heart and lungs. The heart weighed 500 Gm. and showed changes characteristic of hypertensive cardiovascular disease. The left ventricle was hypertrophied, measuring 15 mm. in thickness at the base. The coronary arteries were patent but their lumina were focally narrowed by fibrous and fatty plaques. Microscopic examination revealed focal myocardial fibrosis without evidence of active myocarditis.

The lungs were hemorrhagic, subcrepitant, and together weighed 1,600 Gm. The mucosa of the trachea and bronchi was markedly hyperemic. The lung parenchyma

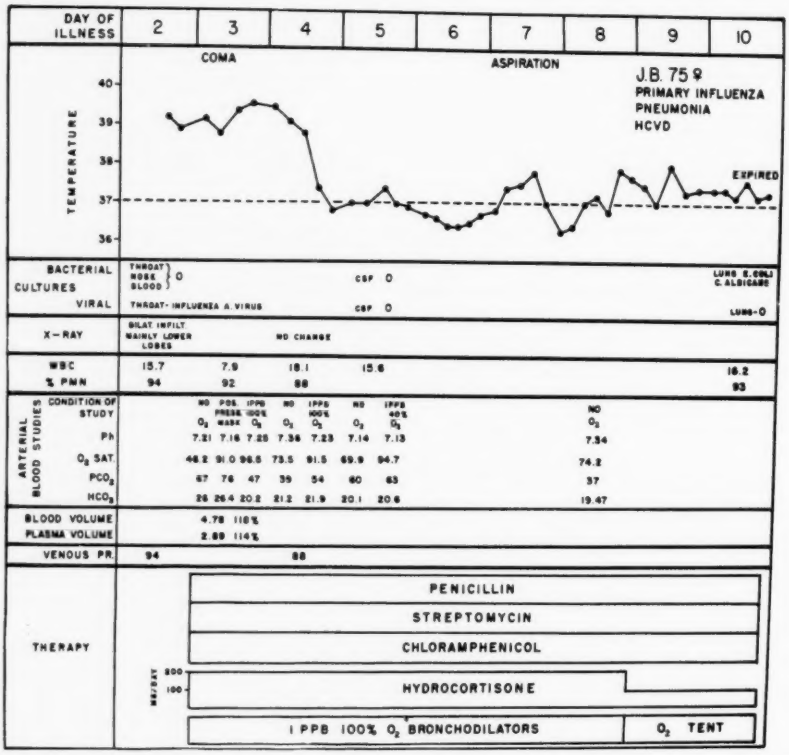

FIG. 23. COURSE OF ILLNESS IN PATIENT J. B., A 75 YEAR OLD WOMAN WITH PRIMARY INFLUENZA VIRUS PNEUMONIA SUPERIMPOSED UPON HYPERTENSIVE HEART DISEASE

was dark red to purple, and a frothy fluid could be readily expressed from the cut surfaces. Numerous nodular areas of consolidation ranging up to 1 cm. across were present in the entire right lung and to a lesser degree in the left lower lobe. Elsewhere the lung was well aerated.

Microscopic examination showed that only the mucosa in the deeper folds of the trachea remained, and even here the normal mucosa was replaced by low cuboidal cells. The submucosal vessels were markedly hyperemic and the interstitial tissues were edematous and infiltrated with moderate numbers of lymphocytes, plasma cells and a few neutrophils. The mucosa of the small bronchi was frequently lined by squamous epithelial cells which showed marked variation in size, shape and staining quality. A number of these cells were in mitosis.

Throughout the lung parenchyma the alveolar capillaries were engorged with red blood cells. The alveolar spaces contained a pink, granular precipitate and varying amounts of fibrin. Focal hemorrhages were present, and in many areas hyaline membranes covered the walls of the alveoli and alveolar ducts. In the upper lobes there were moderate numbers of histiocytes and a few neutrophilic leukocytes and lymphocytes within the alveolar spaces. In the lower lobes all of these changes were more marked. In addition, the small bronchi and many of the alveolar spaces contained masses of polymorphonuclear leukocytes. In some of the small bronchi aspirated muscle fibers and other food particles were demonstrated. In these areas gram-positive cocci and budding yeast-like fungi were seen. Permission to examine the brain was not obtained.

Bacteriologic cultures obtained from the lungs post mortem yielded a few colonies of *E. coli* and moderate numbers of *Candida albicans*. Influenza virus was not recovered from the lung tissue.

Comment. The postmortem findings in this case were those of pneumonia characterized by hemorrhage, edema and hyaline membrane formation. In addition, there was evidence of aspiration pneumonia which was confined to certain sections of the lower lobes. Influenza A virus was isolated from the throat during life but was not isolated from the lung post mortem. It is probable that the failure to recover the virus at autopsy was related to the prolonged period (11 days) between the onset of illness and death (7).

The early recovery of influenza virus from the throat, the clinical syndrome and the postmortem findings were suggestive of an influenza virus pneumonia complicated preterminally by an aspiration pneumonitis. The possibility of concomitant encephalitis was entertained, but influenza virus could not be recovered from cerebrospinal fluid, and other examinations of the spinal fluid were unremarkable. Postmortem examination of the brain was not permitted.

6. A. C., NYH 768041. A 66 year old white man was admitted on Oct. 19, 1957, with increasing respiratory difficulties of one day's duration. This patient had known arteriosclerotic heart disease and a history of two previously documented myocardial infarcts. One day prior to admission he had several shaking chills and developed fever, sore throat, muscle aches and a cough productive of greenish sputum. Progressive respiratory distress led to his admission.

Physical examination on admission revealed an acutely ill man with extreme dyspnea and intense cyanosis of the lips and nailbeds. Temperature was 39.2° C., pulse rate 124 per minute, respirations 40 per minute, and blood pressure 170/100 mm. Hg. Inspiratory rales were heard throughout both lung fields. There was no evidence of pulmonary consolidation. The remainder of the physical examination was within normal limits.

Laboratory studies revealed a white blood cell count of 19,000 cells per cu. mm. with 72 per cent mature polymorphonuclear cells and 14 per cent band forms. The hematocrit was 56 per cent. Urinalysis showed a specific gravity of 1,024, a 4 plus test for protein, 25 to 50 red blood cells and 3 to 8 white blood cells per high power microscopic field. The erythrocyte sedimentation rate was 15 mm. per hour; the serum carbon dioxide combining power was 22 mM per L. Serial electrocardiograms showed no change from previous tracings.

Sputum smears showed no predominance of any bacterium, and sputum and throat cultures yielded only normal pharyngeal flora. Influenza virus with the antigenic characteristics of the Asian strain was recovered from a throat washing. An admission chest roentgenogram showed generalized cardiac enlargement and extensive pulmonary infiltrates which radiated peripherally from the hilar region—a finding consistent with bilateral pulmonary edema or diffuse pneumonitis.

Treatment was instituted with oxygen via positive pressure mask, intravenous aminophyllin, diuretics, digitalis and morphine sulfate. A phlebotomy of 400 ml. of blood was performed. Penicillin, 2.4 million units; streptomycin, 2 Gm.; and erythromycin, 2 Gm., were given daily. Because of continuing cyanosis, the IPPB mask was substituted for the positive pressure mask. The patient's blood pressure fell to 80/40 mm. Hg and levarterenol was added to his regimen. Over the next 24 hours the patient improved markedly. The respiratory rate declined to 30 per minute and cyanosis disappeared. Because the hypotension appeared related to use of oxygen under pressure, the regimen was altered so that 30 minutes of intermittent positive pressure breathing every two hours was alternated with periods in an oxygen tent. The patient's blood pressure stabilized at 130/80 mm. Hg and levarterenol was discontinued. A chest roentgenogram taken 36 hours after admission showed significant improvement.

On the third hospital day the patient had a right sided convulsion followed shortly by a more severe left sided

seizure. Following the seizures blood pressure was unobtainable and inspiratory and expiratory rales and rhonchi were heard throughout the chest. The patient expired eight hours after his initial convulsion.

Findings at autopsy (Autopsy No. 17274). Significant postmortem findings were confined to the heart and lungs. The heart weighed 490 Gm. and showed changes typical of hypertensive and arteriosclerotic cardiovascular disease. There was diffuse sclerosis of the entire coronary system with marked narrowing of the lumina in many areas. Both the right coronary and left circumflex coronary arteries were completely occluded close to their origin. Microscopic sections of the heart revealed extensive fibrosis but no evidence of acute necrosis or myocarditis.

The trachea and major bronchi were extremely hyperemic. The lungs weighed 1,850 Gm. The parenchyma was firm, subcrepitant and dark red, and the cut surfaces exuded a small amount of pink frothy fluid. The right and left lower lobes were consolidated, dark red and airless.

On microscopic examination the mucosa of the trachea was largely denuded or covered by low cuboidal cells, but in some areas normal ciliated cells were present. The submucosal vessels were markedly hyperemic and the interstitial tissue was infiltrated with moderate numbers of lymphocytes and plasma cells and a few neutrophilic leukocytes.

Marked vascular congestion and focal hemorrhages were prominent in the upper lobes, and there were large amounts of pink granular material and fibrin within the alveolar spaces. Histiocytes containing hemosiderin or carbon granules and moderate numbers of neutrophilic leukocytes were also present in the alveolar spaces. Hyaline membranes covered many of the alveolar walls of the upper and right middle lobes.

Congestion of the alveolar capillaries and intra-alveolar edema were more marked in the lower lobes, and in addition the alveoli contained large numbers of polymorphonuclear leukocytes and fibrin. In these lobes the bronchi were frequently ulcerated, and the lumina contained moderate numbers of neutrophilic leukocytes.

Changes suggestive of encephalitis were not seen in sections of the brain.

Bacteria were not seen in sections of the lungs stained by the Gram and Giemsa methods. One postmortem culture yielded two colonies of coagulase negative *Staphylococcus albus* but other cultures were sterile. Asian influenza A virus was recovered in a concentration of 10^2 egg infectious units (EID_{50}) per Gm. of lung tissue.

Comment. Initially, this patient was believed to have acute pulmonary edema of cardiac origin. His early response to therapy for congestive failure appeared to support this possibility. However, the history obtained later of typical influenza followed by respiratory distress, the high fever, leukocytosis and absence of electrocardiographic changes suggested that influenza virus infection was responsible for the syndrome observed. The subsequent isolation of an influenza virus from the throat and lungs, the absence of bacterial pathogens and the characteristic findings at autopsy indicated that this patient had developed a fulminating influenza virus pneumonia.

III. Pneumonia of combined influenza virus and bacterial origin

Nine patients in the current series had evidence of a progressive bilateral pneumonitis with focal signs of consolidation in which both influenza virus and bacterial pathogens were implicated. The following four cases illustrate this syndrome.

1. E. R., NYH 389976. A 35 year old housewife was admitted on Oct. 17, 1957, with fever, cough and chest pain. There was a past history of asymptomatic rheumatic mitral stenosis. The patient was six and one-half months pregnant. The night before admission she developed a scratchy throat and mild generalized myalgia. At 1:00 a.m. on the day of admission she awoke with headache, experienced two shaking chills, developed left anterior chest pain and noted a cough which was productive of bloody sputum.

Admission physical examination revealed an extremely ill, dyspneic woman who was slightly cyanotic. The temperature was 39.5° C., the heart rate 120 beats per min-

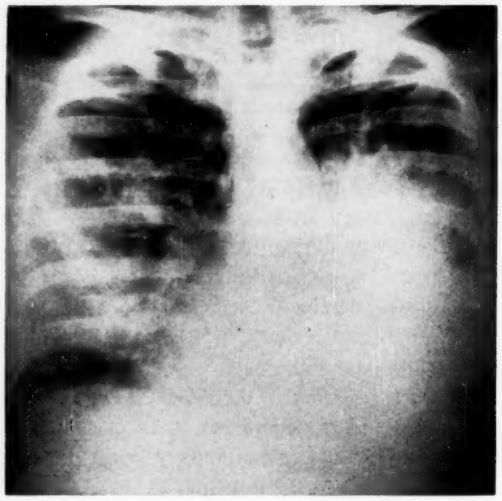

FIG. 24. ADMISSION CHEST ROENTGENOGRAM ON PATIENT E. R., A 35 YEAR OLD WOMAN WITH CONCOMITANT INFLUENZA VIRUS AND PNEUMOCOCCAL PNEUMONIA SUPERIMPOSED UPON RHEUMATIC HEART DISEASE WITH MITRAL STENOSIS AND PREGNANCY

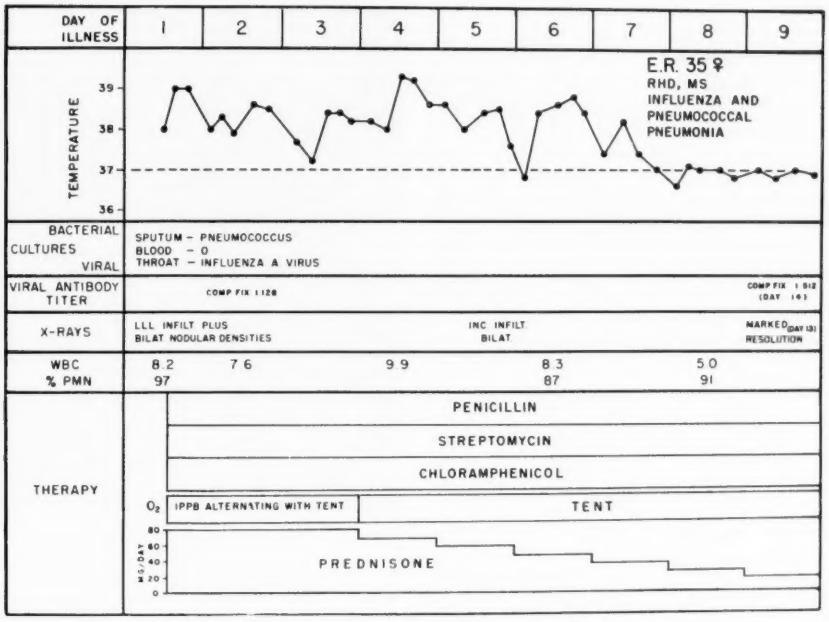

FIG. 25. COURSE OF ILLNESS IN PATIENT E. R.

ute, respirations 44 per minute, and blood pressure 100/60 mm. Hg. No venous distention was present. Classic signs of consolidation were noted over the entire left lower lobe. The heart was slightly enlarged to percussion, and a loud presystolic apical murmur was present. The remainder of the physical examination was within normal limits.

Laboratory studies showed the white blood cell count to be 8,200 cells per cu. mm. with 80 per cent mature polymorphonuclear cells and 17 per cent band forms. The erythrocyte sedimentation rate was 17 mm. per hour. Venous pressure, Decholin® circulation time and an electrocardiogram were normal. Examination of stained sputum smears showed many gram-positive diplococci, and sputum cultures yielded many pneumococci. A chest roentgenogram (Figure 24) revealed homogeneous density throughout the left lower lung field with nodular densities extending from the right hilum.

The course of illness is illustrated in Figure 25. The patient was placed on penicillin, streptomycin and chloramphenicol. Prednisone, 80 mg. daily, was administered by mouth. Oxygen was administered by tent and the IPPB mask. Physical signs of lobar consolidation cleared rapidly; however, the patient remained exhausted, tachypneic and continued to have a fatiguing cough productive of bloody sputum. Repeated chest roentgenography revealed that the diffuse, nodular bilateral infiltrate had increased as the lower lobe infiltrate had subsided. Recovery was slow, the patient remaining febrile until the eighth hospital day. A chest roentgenogram taken 11 days after admission showed marked resolution of the bilateral pneumonic process, but residual bilateral nodular infiltrates were still present (Figure 26). She was discharged without abnormal findings 22 days after admission.

Influenza A virus of the Asian type was recovered from a throat washing obtained on admission. Studies of acute and convalescent serum revealed a fourfold rise in complement fixing antibodies diagnostic of influenza.

Comment. This patient with influenza also had single lobe pneumococcal pneumonia which responded to adequate antimicrobial therapy. However, fever, respiratory distress and hemoptysis persisted for more than a week and symptomatic improvement was slow. Chest roentgenography showed clearing of the lobar infiltrate but progression of a bilateral diffuse infiltrate believed to be indicative of viral involvement. This patient was believed to have combined pulmonary infection caused both by influenza virus and the pneumococcus.

2. A. Z., NYH 781886. A 21 year old woman was admitted on Nov. 8, 1957, because of profound respiratory distress. Three days prior to admission she had developed a sore throat, myalgia, bifrontal headache, a dry cough and fever to 103° F. (oral). She was seen by a physician who noted no respiratory distress or abnormalities on physical examination of the chest. The night

prior to admission she developed pleuritic right chest pain, tachypnea and dyspnea. On the morning of admission her respiratory distress became increasingly severe. When seen by her physician she was markedly cyanotic and audible bubbling sounds could be heard at considerable distance from the patient.

Physical examination on admission revealed a critically ill, anxious, dyspneic woman who was intensely cyanotic. Her temperature was 40.3° C., respiratory rate 60 per minute, pulse 160 per minute, and blood pressure 130/70 mm. Hg. The conjunctivae and pharynx were moderately injected. Crackling inspiratory rales and harsh breath sounds were noted throughout both lung fields. Expiration was labored and appeared to be obstructed. There was evidence of consolidation of both lower lobes. The remainder of the physical examination was not remarkable.

Initial laboratory studies showed the white blood cell count to be 2,000 cells per cu. mm. with 58 per cent lymphocytes, 8 per cent monocytes, 7 per cent polymorphonuclear cells, 9 per cent band forms, 13 per cent metamyelocytes and 5 per cent myelocytes. Urinalysis gave a 3 plus test for protein. The electrocardiogram showed generalized depression of the RT segments with decreased amplitude of the T waves. The patient's arterial oxyhemoglobin saturation was reduced to 71.1 per cent.

Sputum was grossly bloody and contained large numbers of gram-positive cocci. Hemolytic *Staphylococcus aureus* (international bacteriophage type 80/81) was grown in pure culture from the sputum. This organism was sensitive to erythromycin, chloromycetin, streptomycin and novobiocin, but resistant to penicillin and the tetracyclines. The Asian strain of influenza A virus was recovered from throat washings. The admission chest roentgenogram, shown in Figure 27, revealed dense bilateral lower lobe infiltrates with scattered nodular densities present in the central areas of both lung fields.

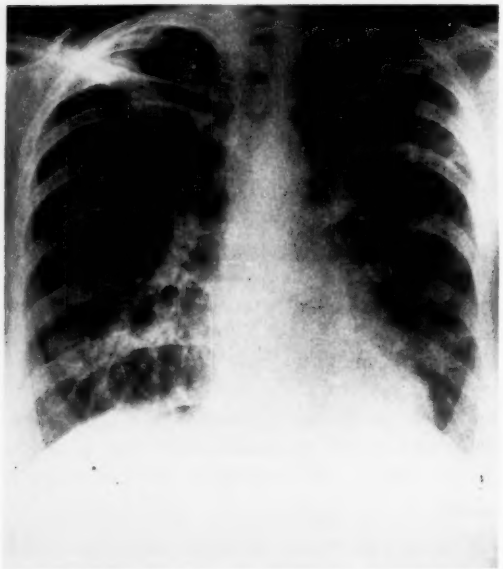

FIG. 26. CHEST ROENTGENOGRAM ON PATIENT E. R. TAKEN ON DAY 11 OF HOSPITALIZATION

Despite virtually complete clearing of the left lower lobe infiltrate, patchy nodular infiltrates remain visible in all lobes.

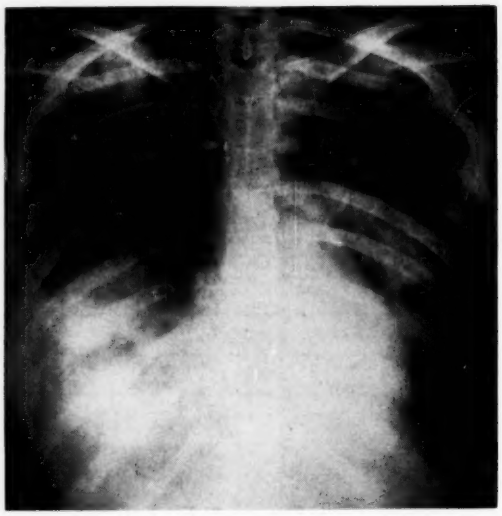

FIG. 27. ADMISSION CHEST ROENTGENOGRAM ON PATIENT A. Z., A 21 YEAR OLD WOMAN WITH INFLUENZA VIRUS AND STAPHYLOCOCCAL PNEUMONIA

The patient was given oxygen through a positive pressure oxygen mask, and administration of erythromycin, dihydrostreptomycin and chloromycetin, 2 Gm. each day, were started. Hydrocortisone, 100 mg. every 12 hours, was injected intravenously, and prednisone, 100 mg. daily, was given by mouth. A summary of the patient's course is depicted in Figure 28.

Over the first four days in the hospital the patient showed moderate improvement. Oxyhemoglobin saturation rose to 93.9 per cent with use of the IPPB mask. Expiratory difficulties appeared relieved with alternating aerosols of Alevaire® and epinephrine, and temperature fell to below 39° C. Nevertheless, signs of consolidation persisted, and she remained cyanotic and tachypneic when oxygen therapy was discontinued.

On the fifth hospital day the patient developed high tracheal obstruction which required tracheotomy and vigorous suctioning. Following this episode her condition worsened rapidly. There were numerous episodes of transient obstruction of various lobes by thick purulent mucus, and her temperature rose to 40 to 41° C. A marked respiratory acidosis developed with the arterial pCO_2 rising to 78 mm. Hg. The administration of acetazolamide, 1.0 Gm. daily, was associated with the return

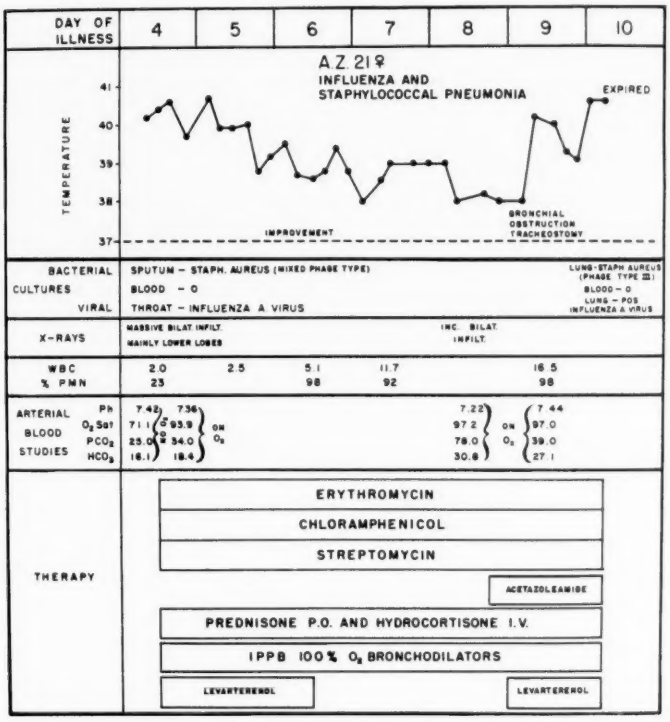

FIG. 28. COURSE OF ILLNESS IN PATIENT A. Z.

of arterial blood pCO_2 and pH to normal, but there was no improvement in the patient's clinical course. The onset of bloody diarrhea was associated with the recovery of hemolytic *Staphylococcus aureus* from stool cultures. On the sixth hospital day blood pressure fell to shock levels and the patient died.

Findings at autopsy (Autopsy No. 17315). Autopsy was limited to the removal of sections of organs through an abdominal incision. The lungs were obviously heavy and the lower lobes were nodular and firm. On section the parenchyma was diffusely hemorrhagic and airless, and small abscesses containing soft, yellow, purulent material were found in all lobes.

Microscopic examination revealed widespread ulceration of the bronchial mucosa together with extensive alveolar edema and hemorrhage. Many of the alveoli and alveolar ducts were covered with a thick hyaline membrane. Interstitial tissues were edematous and contained numerous polymorphonuclear leukocytes. As illustrated in Figure 10, the abscesses showed central necrosis and contained many colonies of cocci. Microscopic sections of the heart were not remarkable.

Quantitative bacterial cultures of the right upper and left lower lobes demonstrated a pure growth of hemolytic *Staphylococcus aureus* in concentrations of 5×10^8 microorganisms per Gm. Bacteriophage typing revealed that these microorganisms were of phage type III (53 VA 4). This strain was resistant to more than 50 μg. of all antimicrobials except novobiocin. Influenza A virus of the Asian strain was demonstrated in a concentration of 10^2 EID_{50} per Gm. in lung tissue.

Comment. This young patient developed a rapidly fatal bilateral pneumonia. Both staphylococci and influenza virus were isolated from the sputum and the lungs at autopsy. During her hospitalization of six days, a drug-sensitive staphylococcus was replaced by a staphylococcus of different phage type which was insensitive to all antimicrobial agents used in treatment. It seemed probable that this microorganism entered the respiratory tract via tracheostomy tube during the last 36 hours of life.

3. R. C., NYH 653327. A 31 year old white woman was admitted on Oct. 23, 1957, with fever, cough and respiratory distress. The patient had known rheumatic heart disease and had undergone a mitral commissurotomy four and one-half years prior to admission. In recent months she had noted increasing fatigue and exertional dyspnea. Four days prior to admission the patient de-

veloped an illness characterized by chills, fever and photophobia. Two days prior to admission she noted increasing cough, chills, fever and increasing dyspnea.

Physical examination disclosed an acutely and chronically ill cyanotic woman with moderate dyspnea. The temperature was 40.0° C., respirations 28 per minute, heart rate 130 beats per minute, and blood pressure 98/65 mm. Hg. The neck veins were moderately distended. Examination of the chest revealed bilateral moist inspiratory rales over both lower lung fields. Sticky inspiratory rales and bronchial breath sounds were detected over the right middle lobe. The expiratory phase of respiration was moderately prolonged. The heart was moderately enlarged, and loud systolic and diastolic murmurs were heard at the apex. The liver was enlarged to 5 cm. below the right costal margin and was tender. No peripheral edema was present.

Pertinent laboratory studies revealed a white blood cell count of 10,200 cells per cu. mm. with 67 per cent mature polymorphonuclear cells, 20 per cent band forms, and 2 per cent metamyelocytes. The sedimentation rate was 24 mm. per hour. Electrocardiograms showed sinus tachycardia and evidence of right ventricular hypertrophy and "digitalis effect." The venous pressure was 150 mm. saline at the level of the right auricle.

Sputum cultures yielded many colonies of hemolytic *Staphylococcus aureus* and pneumococci. The staphylococcus isolated was sensitive to the tetracyclines, chloramphenicol and erythromycin but was resistant to 3.25 units of penicillin. Chest roentgenography revealed diffuse, patchy densities radiating from the hilar areas bilaterally.

It was believed that the patient had chronic congestive heart failure and combined influenza virus and bacterial pneumonia. Treatment was initiated with digitoxin, mercurial diuretics and oxygen via the IPPB mask. Erythromycin and chloramphenicol, 2 Gm. daily, were given by mouth.

On this program the venous pressure returned to normal, but pulmonary signs failed to improve. Prednisone, 60 mg. daily, was started at this time. Dramatic improvement followed the initiation of steroid therapy. One week later prednisone was slowly withdrawn only to be followed by the return of fever, fatigue and dyspnea. Chest roentgenograms showed some clearing, but a diffuse reticular infiltrate remained. Prednisone was reinstituted and again there was rapid improvement and defervesence. Attempts were again made to withdraw the steroids, but again the patient's symptoms and fever returned and prednisone was therefore continued. The patient pursued a gradual downhill course characterized by fever, anemia and increasing heart failure, and she expired on the forty-ninth hospital day.

Study of paired serums obtained on the sixth and thirty-eighth day of illness showed an eightfold rise in influenza A complement fixing antibody.

Findings at autopsy (Autopsy No. 17357). The principal autopsy findings were confined to the heart and lungs. The heart weighed 380 Gm. and the myocardium was remarkably flabby. The mitral valve orifice was narrowed to a slit-like opening, and the mitral valve leaflets were thickened by fibrous tissue and focal calcification. The chordae tendineae were thickened and shortened. The walls of the coronary arteries were thin and their lumina patent. Microscopically there was an extensive infiltration of polymorphonuclear leukocytes associated with focal necrosis of myofibers throughout the heart. Aschoff bodies were not present. The blood vessels throughout the heart appeared normal.

The lungs together weighed 1,280 Gm. and the middle and lower lobes of the right lung were dark red and firm. Sections of the lung revealed marked passive hyperemia with extensive hemosiderin deposits within many mononuclear cells together with much alveolar hemorrhage and edema of recent origin. In some areas there was interstitial fibrosis and fibrosis of alveolar walls. Many small arteries, arterioles and capillaries in the right middle and lower lobes were necrotic and these areas were infiltrated with polymorphonuclear leukocytes. Cultures of lung tissue revealed a few colonies of coagulase positive staphylococci, *Candida albicans,* and *E. coli.* Influenza virus could not be obtained by inoculation of lung suspensions into embryonated eggs.

Comment. This patient with severe rheumatic heart disease developed influenza which was followed by bacterial pneumonia and congestive heart failure. Although there was an initial improvement, the subsequent course was one of fever, increasing congestive failure and anemia.

Autopsy was performed 53 days after the onset of her illness. A necrotizing myocarditis and pulmonary arteritis and arteriolitis were superimposed on chronic rheumatic deformities of the mitral valve. Influenza virus could not be recovered from the lung tissues.

This picture was strikingly different from that noted in other patients in this series. The prolonged course characterized by cardiac failure and anemia and the failure to demonstrate the influenza virus or the usual pulmonary changes associated with influenza suggest that the arteriolitis and myocarditis were perhaps of non-influenzal etiology.

4. B. P., NYH 73753. A 61 year old woman was admitted on Jan. 26, 1958, because of hemoptysis and dyspnea. The patient had been in good health until three days prior to admission when she noted the onset of fever, sore throat and shaking chills, followed in the next 24 hours by headache, nonproductive cough and generalized myalgia and arthralgia. She improved transiently but on the day of admission developed wheezing and respiratory distress, and her cough became productive of bloody sputum.

Physical examination revealed an acutely ill, cyanotic woman in marked respiratory distress. The admission

temperature was 40.4° C., the heart rate 122 beats per minute, respirations 60 per minute, and blood pressure 100/60 mm. Hg. There was no venous distention. Examination of the chest revealed dullness to percussion over the left lower thorax posteriorly. Tubular breath sounds were heard in the area of dullness. Throughout the rest of the lung fields breath sounds were harsh, and grating expiratory rales and rhonchi were present. Examination of the heart revealed a sinus tachycardia and an accentuated pulmonic second sound. Physical examination was otherwise within normal limits.

Marked leukopenia was revealed by laboratory studies. The total leukocyte count was 1,400 cells per cu. mm. with 86 per cent lymphocytes and 14 per cent polymorphonuclear cells. Hemoglobin and hematocrit determinations and a platelet count were normal. Granulocytic maturation arrest and a moderate increase in eosinophiles were evident in a bone marrow aspirate. A venous pressure determination and electrocardiogram were normal. The venous carbon dioxide combining power was 16 mM per L.

Myriads of gram-positive cocci, red blood cells and leukocytes were seen in Gram stained smears of the sputum. Cultures yielded a pure growth of hemolytic *Staphylococcus aureus* (bacteriophage type I) which was sensitive to all antimicrobials tested. Blood cultures were sterile. A chest roentgenogram obtained on admission showed a dense left lower lobe infiltrate and a bilateral diffuse increase in hilar markings (Figure 29). Influenza virus (Asian strain) was recovered from the admission throat washing.

Antimicrobial therapy was initiated with chloramphenicol, 2 Gm. per day; streptomycin, 1 Gm. per day; and procaine penicillin, 2.4 million units per day. Oxygen was administered via the IPPB mask. Shortly after admission, blood pressure fell to 70/40 mm. Hg and intravenous norepinephrine was started. Hydrocortisone, 100 mg., was given intravenously. Despite this therapy, illness progressed. Twelve hours after admission arterial blood gas studies without administration of supplemental oxygen disclosed an oxyhemoglobin saturation of 70.5 per cent and carbon dioxide tension of 27 mm. Hg. The serum bicarbonate concentration was 9.63 mM per L., and the arterial blood pH was 7.18 units.

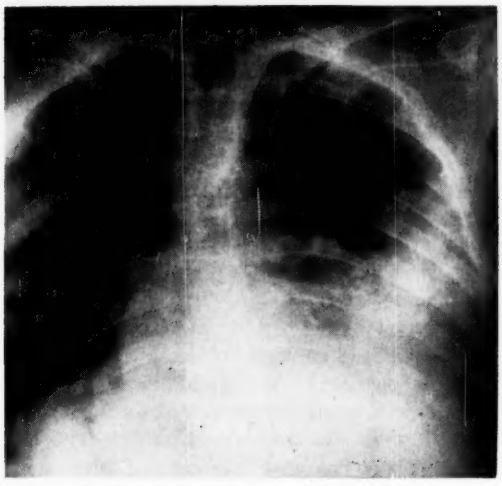

FIG. 29. ADMISSION CHEST ROENTGENOGRAM ON PATIENT B. P., A 61 YEAR OLD WOMAN WITH CONCOMITANT INFLUENZA VIRUS AND STAPHYLOCOCCAL PNEUMONIA

Penicillin dosage was increased to 40 million units intravenously per 24 hours given as a continuous slow infusion, but respiratory distress progressed relentlessly, and the patient died 20 hours after admission.

Findings at autopsy (*Autopsy No. 17449*). The mucosal surfaces of the larynx, trachea and major bronchi were hyperemic and granular and the lumina contained much bloody fluid. The lungs were heavy and hemorrhagic and together weighed 1,920 Gm. The basilar portions of the upper lobes, the entire right middle lobe and both lower lobes were noncrepitant, firm and dark red.

In the upper lobes, the alveolar capillaries were markedly hyperemic. Many of the alveolar lumina were filled with a pink, granular precipitate, and in some areas alveolar hemorrhage was prominent. The ciliated epithelium of many of the small bronchi appeared normal, but their lumina contained cellular debris, neutrophilic leukocytes, red blood cells, edema fluid and many gram-positive cocci. In one area the inflammatory process was associated with necrosis of alveolar walls. All of these changes were much more marked in the lower lobes, and hemorrhage, edema and abscess formation were widespread. Huge numbers of gram-positive cocci were readily identified within the lung parenchyma. Careful search failed to reveal hyaline membranes in any lobes of the lungs. The remainder of the organs, including the brain, were grossly and microscopically unremarkable.

A pure growth of hemolytic *Staphylococcus aureus* was cultured in high titer from all lobes. Staphylococci in concentrations of 4×10^4 microorganisms per Gm. were grown from the left upper lobe. The right lower lobe and left lower lobe contained 4×10^8 viable staphylocci per Gm. The staphylococci isolated were of the same phage type and antimicrobial sensitivity as the strain originally isolated from the sputum. The Asian strain of influenza A virus was recovered from the lungs in concentrations of $10^{2.7}$ EID_{50} per Gm.

Comment. This patient had a typical influenzal illness followed in three days by a fulminating pneumonia which resulted in death. Both influenza virus and hemolytic *Staphylococcus aureus* were isolated from lung tissue post mortem. Leukopenia and granulocytopenia accompanied the illness. Despite the susceptibility of the staphylococcus to the antimicrobials administered, the patient died shortly after admission to the hospital.

RECAPITULATION

The present paper reports observations on 33 patients with proven influenza A infection who developed pulmonary complications during their illness. On the basis of detailed clinical and laboratory study, four clinical syndromes were recognized:

1. Influenza with physical signs of lung involvement but without roentgenographically detectable pulmonary infiltrates.

2. Focal bacterial pneumonia following influenza.

3. Influenza associated with a diffuse pulmonary process produced by influenza virus infection alone.

4. Influenza associated with both focal and diffuse pulmonary signs resulting from concomitant bacterial and influenza virus infection.

Influenza with physical signs of lung involvement without roentgenographic pulmonary infiltrates

Three patients developed scattered rales and wheezes over the lower lung fields without evidence of bacterial infection or roentgenographic infiltrates. Each of these patients had underlying disease producing pulmonary hypertension. These patients recovered without antimicrobial therapy in a manner generally similar to that seen in uncomplicated influenza.

Transient lower respiratory tract signs without changes in chest X-ray films have been reported to occur in occasional patients with influenza and have been considered to represent bronchial and bronchiolar involvement (17, 18). It seems probable that the relative infrequency of this pulmonary syndrome in our series was the result of an admission policy in force during the epidemic which suggested the hospitalization of influenza patients only when pulmonary signs were accompanied by roentgenographic pulmonary infiltrates.

Focal bacterial pneumonia following influenza contrasted with the syndrome of diffuse primary influenza virus pneumonia

Fifteen of the 33 patients had definite evidence of *bacterial pneumonia* arising sometime after the onset of influenza. Six patients manifested a *severe, diffuse pulmonary syndrome without apparent bacterial infection* which appeared to result from influenza virus infection *per se*. These two syndromes differed sharply from one another and could be separated clinically with increasing certainty as the epidemic proceeded. These differences deserve enumeration.

1. The host setting in which disease arose was different in the two syndromes. Focal bacterial pneumonia often arose in previously healthy individuals. In this group the numbers of male and female patients were approximately equal.

In contrast, all six of the patients with the diffuse abacterial pulmonary syndrome had underlying heart disease. Rheumatic heart disease with mitral valve involvement was present in four. Five of the six patients were males.

2. The progression of the illnesses differed. Patients who presented with what was subsequently proved to be bacterial pneumonia usually experienced a longer interval between the onset of influenza symptoms and hospitalization which averaged 3.8 days. Ten of the 15 patients with bacterial pneumonia had noted a period of relative freedom from symptoms following influenza before symptoms suggesting bacterial infection became apparent. Chills, fever, cough productive of purulent or bloody sputum and pleural pain were the principal complaints upon admission.

In contrast, all patients presenting with diffuse influenza virus pneumonia had had illness of short duration. In these patients typical influenza symptoms were followed in less than 24 hours by progressive dyspnea. Profound respiratory distress was the universal complaint upon admission. Pleural pain was not present.

3. Findings on physical examination were different in the two groups. The majority of patients with focal bacterial pneumonia had high fever, grunting respirations and typical signs of focal consolidation over one or more lobes. Cyanosis or severe respiratory distress was rare.

In contrast, patients with diffuse, abacterial pneumonia had no signs of local consolidation. Chest findings were bilateral and diffuse, and were characterized by difficulties in air exchange, rales, inspiratory and expiratory wheezes and rhonchi. Cyanosis was uniformly present and respiratory distress was extreme.

4. Certain laboratory studies aided in differentiating these two syndromes. a) Many polymor-

phonuclear leukocytes and masses of bacteria were observed in smears of sputum obtained from patients with bacterial pneumonia. Pathogens known to produce pneumonia were readily cultured from the sputum. In contrast, sputum smears obtained from patients with influenza virus pneumonia revealed only sparse bacteria, and sputum cultures consistently failed to yield significant pathogenic microorganisms.

b) Differences were apparent in the incidence of recoveries of influenza virus from throat washings. No influenza virus was obtained from 13 patients with bacterial pneumonia in whom virus isolation was attempted. In contrast, influenza virus was recovered from three of four patients with primary influenza virus pneumonia from whom throat washings were obtained.

c) Roentgenographic findings differed in patients with secondary bacterial and primary influenza virus pneumonia. Chest X-rays on patients with bacterial pneumonia commonly showed typical focal infiltrates confined to one or more lobes. In patients with influenza virus pneumonia, a diffuse, fanning, bilateral, perihilar infiltrate, indistinguishable from the roentgenographic picture observed in cardiac pulmonary edema was a characteristic finding.

5. The course of illness was different in patients with secondary bacterial and primary influenza virus pneumonia. Patients with focal bacterial pneumonias responded promptly to antimicrobial therapy in a manner similar to that ordinarily seen in primary bacterial pneumonias. The mortality was low in this group. One of 15 patients died with what appeared to be a complication unassociated with her influenza.

In contrast, patients with diffuse abacterial pneumonia failed to respond to the administration of large doses of antimicrobial drugs. Hypoxia, respiratory distress and respiratory and metabolic acidosis progressed relentlessly despite therapy. Five of the six patients with this syndrome died.

Combined influenza virus and bacterial pneumonia

Nine patients with pulmonary infiltrates had an illness which was characterized by certain features of both focal bacterial pneumonia and diffuse influenza virus pneumonia. In these patients the duration of illness prior to hospitalization was variable. Respiratory distress was a universal complaint on admission. Both focal and diffuse findings were usually present on physical and roentgenologic examination. Large numbers of pneumococci, staphylococci or *Hemophilus influenzae* were isolated from the sputum, and influenza virus was simultaneously present in seven of nine throat washings obtained. This syndrome, in common with primary influenza virus pneumonia, was associated with a significant mortality. Four of the nine patients died during the course of their illness. Findings during the acute illness in the two patients examined post mortem were similar to those noted in primary influenza virus pneumonia, but in addition, histologic and cultural evidence of an extensive necrotizing staphylococcal pneumonia was present.

Other laboratory features of pneumonia associated with influenza

Certain laboratory studies did not aid in differentiating the pulmonary syndromes observed. In all syndromes patients demonstrated a leukocytosis with an increase in absolute numbers of granulocyte forms. Erythrocyte sedimentation rates were usually normal in patients with diffuse influenza virus pneumonia and high in patients with associated bacterial infection, but too few patients were studied to assess the constancy of this observation.

No evidence of renal or hemopoietic damage or defects in blood clotting mechanisms accompanied influenza. Tests of hepatic function were generally within normal limits during the acute illness except for serum glutamic oxaloacetic transaminase determinations. Transaminase levels were elevated in the majority of patients studied, and the degree of elevation of serum transaminase levels appeared to parallel the severity of the illness. In the absence of other changes suggestive of hepatic or myocardial damage, the significance of this finding remains unclear. Viremia was not demonstrated during the course of influenza.

Consideration of primary influenza virus pneumonia as a specific disease entity

As the belief evolved that pure influenza virus pneumonia represented a definite entity, two questions arose in considering the clinical syndrome

manifested by the six patients described in the present report.

1. Could bacterial infection have been missed in these patients who received large amounts of antimicrobials? This has seemed unlikely. First, bacteria were easily cultured from sputum samples obtained from patients simultaneously admitted with other pulmonary syndromes, while repeated cultures did not yield significant bacterial pathogens from the sputum of these six patients. Secondly, three of five patients with the diffuse pulmonary syndrome died within 48 hours after institution of therapy without bacteriologic or histologic evidence of infection. Experimental evidence indicates that antimicrobial therapy rarely eradicates microorganisms residing in tissues within this time period. Furthermore, microorganisms were cultured in high concentrations and were readily seen on histologic sections from the lungs of influenza patients with bacterial pneumonias who died after comparable time periods on similar therapy. Thus it seems unlikely that antibiotics induced bacterial sterility in lungs during such short periods of drug administration.

It is conceivable that antimicrobial treatment could eradicate pneumococci because of their tendency to autolyse rapidly when killed. Nevertheless, the diffuse pulmonary syndrome observed, the lack of response to antimicrobials, the presence of distinctive and unusual anatomic findings, and the absence of the pulmonary histologic changes usually seen in pneumococcal pneumonia suggest that this was a most unlikely possibility. Lastly, high concentrations of influenza virus were demonstrated in the lungs of these patients. In three of the four patients, virus concentrations were 20 to 2,000 times those found in patients with influenza who died with obvious bacterial infections.

2. Was it possible that this diffuse syndrome represented acute left heart failure? All of the patients with diffuse abacterial pneumonia had antecedent heart disease, and chest X-ray films were compatible with acute pulmonary edema. There was, however, considerable evidence to indicate that the process observed was not congestive heart failure.

First, four of the six patients had significant pulmonary hypertension secondary to mitral valvular lesions. It might be expected that any additional stress placed on the heart would result in rapid *right* heart failure. Nevertheless, signs of right heart failure were generally absent, and blood volumes and venous pressures were commonly normal. Secondly, there were no physical or electrocardiographic signs to suggest the presence of myocarditis. Third, the institution of vigorous therapeutic measures for acute congestive heart failure produced no significant change in the pulmonary picture. Lastly, the findings at autopsy were not those of heart failure and indicated that a diffuse pulmonary infection was present.

We are thus led to the conclusion that influenza virus infection in the absence of bacterial complications produced a characteristic, severe and usually fatal pneumonic syndrome in certain patients with underlying heart disease.

DISCUSSION

At the outset of the recent pandemic there was little evidence that influenza virus *per se* could cause fatal pneumonia when unattended by bacterial pathogens. The first documented abacterial influenza virus pneumonia, described in 1945 by Finland, Barnes and Samper (19), was associated with an interpandemic infection. The following year a second case report appeared from the same group of investigators (15). In both cases the minimal acceptable criteria for primary influenza virus pneumonia were met; virus was recovered from lung tissue and bacterial pathogens could not be demonstrated (see Table XV). Except for an undocumented reference to another case of apparent abacterial influenza virus pneumonia in 1948 (20), other instances of fatal primary influenza virus pneumonia were not described before the recent pandemic.

The present investigation has documented six cases of influenza virus pneumonia in which bacterial pathogens could not be incriminated. Five of these patients died. Other investigations of the 1957–58 pandemic also present evidence that fatal abacterial influenza may occur. Four of eight fatal pneumonias reported by Giles and Shuttleworth were bacteriologically sterile (21). Details of these cases were not published. In a large prospective study of fatal influenza in the Netherlands, 22 instances of bacterially sterile lungs were reported in 103 patients in whom pulmonary in-

fluenza virus was demonstrated post mortem (22). Three additional cases have been reported recently in which influenza virus was recovered from bacteriologically sterile lungs (23, 24). Thus at the present writing, 31 such cases (excluding the present six) have been reported (Table XV). Although detailed presentations of the clinical manifestations of illness have been few, our experience, supported by the reports cited above, indicate that influenza virus infection *per se* can be a cause of pneumonia and death in man.

Although the view has been widely held that influenza virus infection *per se* does not produce fatal disease, it has long been recognized that influenza epidemics are closely associated with an increased incidence of bacterial pneumonia (25, 26). In the present study, 24 of 33 patients had evidence of superimposed or subsequent bacterial pulmonary infection. In the 1918–19 pandemic, pathogenic bacteria were almost invariably isolated from the lungs of fatal cases of influenza (27–29). Since the initial recognition of influenza virus in 1933 (3) the isolation of *both* influenza virus and bacteria from the lungs of fatal cases has been repeatedly documented (17, 18, 30). In the majority of such fatal pneumonias, the associated bacterial pathogen has been *Staphylococcus aureus* (17, 18, 30 and 31). The frequent isolation of staphylococci and influenza virus from the lungs in fatal influenza (Table XV) has focused attention on the bacterial problem, especially as antibiotic-resistant staphylococci have become increasingly prevalent.

In the absence of firm evidence in the past that influenza virus alone might induce fatal pneumonia, the pathogenic contribution of the virus in combined viral-bacterial infections has often been considered minor. The fact that effective antibacterial drugs were available in the recent pandemic has allowed clearer differentiation of the relative importance of the virus in such combined infections. The present investigation, in which bacteria generally sensitive to antimicrobials were encountered in patients with concomitant viral and bacterial pneumonia, has allowed some clarification of the role of the influenza virus in such combined infections.

Three facts suggest that the severity of the influenza virus infection *per se* may be important in determining the outcome of combined influenza and bacterial pneumonia:

First, it was apparent from the present investigation that patients died from influenza virus infection alone.

Secondly, the diffuse nature of the pulmonary disease observed and the severe respiratory difficulties associated with combined viral-bacterial pneumonia in certain patients were not characteristic of bacterial pneumonia but more closely resembled the signs and symptoms observed in patients with pure influenza virus pneumonia.

Third, certain patients with combined infection produced by influenza virus and antibiotic-sensitive bacteria failed to exhibit an appropriate response to antimicrobial agents. Deaths in some of these patients were clearly not related to a failure of antibacterial therapy but seemed to result from the unmitigated effects of the viral component of the combined infection. In those in this group who survived, the recovery phase was protracted, regardless of the bacterial pathogen involved. This slow response stood in sharp contrast to the response of patients who developed secondary pneumonia following the earlier onset of influenza.

On the basis of this experience the present authors are led to believe that the virus infection does more than merely reduce host resistance to bacterial invasion. It seems probable that the influenza virus *per se* plays an important role in deaths observed in such combined viral-bacterial pneumonias.

The deaths observed in two patients with combined influenza virus and staphylococcal infections were reminiscent of the fatalities attributed to staphylococcal pneumonia in 1918–19 by Chickering and Park (32). The syndrome described by these authors has always been puzzling to students of staphylococcal infection because of the diffuse pulmonary involvement and the lack of pleural pain or empyema so common in most staphylococcal pulmonary infections (33). Our recent experience suggests that the clinical manifestations observed by Chickering and Park may have been determined by influenza virus infection acting in concert with necrotizing bacterial disease rather than staphylococcal infection alone.

The picture of the staphylococcus as an increasingly important co-invader in influenza must be viewed with caution. In the present investigation of living patients, the high incidence of pneumococcal pneumonia contrasts with the low inci-

dence of pneumococci reported in autopsy material from fatal cases of influenza (22). It should be remembered that the antimicrobial treatment of pneumococcal or streptococcal infections is vastly more effective than the treatment of staphylococcal disease. Antimicrobial therapy may thus, in part, select the bacterial agent (staphylococcus) which will result in an influenza fatality, and a spurious etiologic relationship may be inferred by study of postmortem materials alone.

The present study emphasizes the importance of underlying disease in the pathogenesis of pulmonary complications of influenza. Twenty-one of the 30 patients with serious pulmonary complications, including nine of the 11 who died, had underlying illness or were pregnant. This correlation was even more striking in patients with diffuse virus pneumonia with or without pre-existing disease. In this group, 13 of the 15 patients had pre-existing disease. This experience is in general accord with the observations of others reported during the last two decades. Table XV summarizes published reports of fatal cases of influenza in which virus with or without associated bacterial pathogens was demonstrated in the lung. In 14 of these cases adequate data were available to determine whether or not underlying disease was present before the onset of influenza. Six of these 14 patients clearly had preceding disease. In two other reported cases the data provided suggest the possibility of antecedent disease. Of the remaining six patients without manifest antecedent

TABLE XV

Fatal cases of influenza in which virus was demonstrated in the lung

Reference	Date	Patient	Age	Sex	Underlying disease*	Bacterial culture†		Isolation of influenza virus		Comment
						Sputum	Lung	Throat wash.	Lung	
1. Scadding (17)	1937	K. O.	16	F	? RHD at 12	NR	Staph.	NR	A	
2. Stuart-Harris, Andrewes and Smith (18)	1938	A. L. E.	?	?	No details	NR	Staph.	ND	A‡	
		O. A. K.	?	?	No details	NR	Staph.	ND	A‡	
		W. A. N.	?	?	No details	NR	Staph.	ND	A‡	
3. Stokes and Wolman (39)	1940		20	F	Furunculosis	NR	Staph.	NR	A	
4. Himmelweit (30)	1943		55	M	None	NR	Staph.	NR	B	
5. Finland, Barnes and Samper (19)	1945	A–1	18	F	None	β strep. staph.	β strep. staph.	NR	A	
		A–2	39	M	CAD	0	Alpha strep.	NR	A	This organism is an unlikely pathogen
		A–3	61	M	ASCVD	Pneumo. I	Pneumo. I	NR	A	
6. Parker, Jolliffe, Barnes and Finland (15)	1946	1	18	F	None	NR	Staph.	ND	A	
		2	34	F	Antecedent cough, fatigue, coincident myocarditis	*N. catarrhalis* staph. (few)	0	ND	A	Abacterial
7. Burnet, Stone and Anderson (40)	1946	3	47	F	No details	ND	Staph.	ND	B	(Reisolated) bronchial washings
8. Maxwell, Ward and Van Metre (20)	1949	P47A1	14 mo.	M	Varicella	NR	ND	NR	A	
		P47A2	7 mo.	M	Meningitis	NR	ND	NR	A	
		P46A1			No details	NR	Pneumo. II	NR	A	
		P47B1			No details	Pneumo. IX	0	NR	B	Possibly abacterial
9. Stuart-Harris and co-workers (26)	1949	4 cases			No details	NR	Staph.—4	NR	A	
10. Mulder and Verdonk (41)	1949	K	27	M	None	Staph. (lung puncture)	Staph.	ND	A‡	Tracheal isolation

* RHD, Rheumatic heart disease; MS, Mitral stenosis; CAD, Coronary artery disease; ASCVD, Arteriosclerotic heart disease.

† NR, Not reported; ND, Not done; Staph., hemolytic Staphylococcus aureus; Strep., Group A hemolytic streptococcus; pneumo., pneumococcus.

‡ Identification of virus only presumptive on basis of data published.

Note—Isolations of virus from 195 fatal cases (43) are not summarized here because the data in this report do not allow conclusions regarding individual cases nor is it stated how many isolations are from pulmonary tissue.

TABLE XV—*Continued*

Reference	Date	Patient	Age	Sex	Underlying disease*	Bacterial culture†		Isolation of influenza virus		Comment
						Sputum	Lung	Throat wash.	Lung	
11. Evans and Evans (42)	1956	1	53	F	No details	Staph.	NR	ND	A	No data on viral isolation
12. Giles and Shuttleworth (21)	1957	8 cases			No details	NR	Staph.—4 0—4	NR	A (Asian)	Four abacterial
13. Hers, Goslings, Masurel and Mulder (22)	1957	103 cases			See comment		Staph.—69 β strep.—1 Pneumo.—3 *H. influ.*—1 *E. coli*—7 0—22		A (Asian)	22 abacterial. Of 10 patients with RHD and MS, nine were abacterial
14. Perrier, Hausser and Paccaud (23)	1957	L. T.	19	M	None	NR	0	NR	A (Asian)	Two abacterial
		H. B.	57	F	None (obesity)	NR	0	NR	A (Asian)	
15. Rock, Braude and Moran (24)	1958		38	F	RHD, MS	*Strep. viridans*, *N. catarrhalis*	0	ND	A (Asian)	Virus reisolated, abacterial
16. Present report	1958	G. H.	49	M	RHD	Rare pneumo., normal flora	0	ND	A (Asian) ($10^{5.3}$)	First four cases abacterial
		C. P.	47	M	RHD	Normal flora, rare pneumo., pseudomonas, proteus	0	ND	A (Asian) ($10^{6.7}$)	Virus reisolated from lungs in five of seven cases
		A. B.	45	M	RHD	Normal flora, few *H. influenzae*, pneumo.	0	A (Asian)	A (Asian) (10^{6})	
		A. C.	66	M	ASCVD	Normal flora	0	A (Asian)	A (Asian) ($<10^{2}$)	
		F. R.	53	M	Myeloma	Pneumo.	0	A (Asian)	A (Asian) ($<10^{2}$)	
		A. Z.	21	F	None	Staph.	Staph. 5×10^{8}/ml.	A (Asian)	A (10^{3}) (Asian)	
		B. P.	61	F	None	Staph.	Staph. 4×10^{8}/ml.	A (Asian)	A (Asian) ($10^{2.7}$)	

disease, four had both staphylococcal and influenza virus lung involvement. Thus only two of the 14 patients reported in adequate detail could be considered to be normal individuals who died solely of uncomplicated influenza virus infection.

The specific association of underlying cardiovascular disease with serious pulmonary complications of influenza was particularly striking in the present study (Table XVI). Fourteen or two-thirds of the 21 patients with an antecedent illness had cardiovascular disease; 10 of the 14 had rheumatic heart disease. If the patients with diffuse lung involvement (with or without bacterial infection) are considered as a separate group, the incidence of underlying cardiovascular disease is even more impressive. Eleven of 15 such patients had known underlying cardiac disease. In seven of these 11, rheumatic heart disease was present.

The impression that individuals with rheumatic heart disease manifest increased susceptibility to pneumonic involvement with influenza virus infection is supported by other observations reported from the pandemic of 1957–58. Rock, Braude and Moran have reported a patient with mitral stenosis who developed fatal abacterial influenza virus pneumonia (24). In the Netherlands study, nine of 10 cases with mitral stenosis had abacterial influenza virus pneumonia (22). Thirty-five of 46 fatal cases with clinical diagnoses of influenza reported by Giles and Shuttleworth had pre-existing disease. In seven of the 35, rheumatic heart disease was present (21).

The prognostic import of underlying rheumatic heart disease was grim with any of the pneumonic syndromes associated with influenza observed in the present study (see Table XVI). The only

death among patients with late bacterial pneumonia following influenza occurred in a rheumatic patient. Of three patients with rheumatic heart disease and concomitant bacterial and viral pneumonia, one died; of four with rheumatic heart disease and primary influenza virus pneumonia, three died. Thus five of 10 rheumatics who developed pneumonia in association with influenza failed to survive.

Thus, the present study suggests not only the importance of *antecedent* disease but specifically the importance of *cardiovascular disease* in the pathogenesis of influenza virus pneumonia. The fact that mitral stenosis was the most common cardiovascular defect suggests the probable importance of hemodynamic rather than general debilitating factors in the genesis of the syndrome. It seems probable that the pulmonary hypertension associated with mitral stenosis may play an important role in the pathogenesis of the hemorrhagic, edematous pneumonia induced by influenza virus.

Certain comparisons of the pandemics of 1918–19 and 1957–58 can be made on the basis of the present experience. The clinical picture of influenza in uncomplicated cases in 1957–58 (5, 6) was remarkably similar to that described in 1918–19 (34). The clinical syndrome of fulminating pneumonia as observed in the present series also closely resembles certain clinical descriptions of fulminant pneumonia in 1918–19 (35, 36).

There were, however, two important differences in the two pandemics: First, the fatality rate was high in 1918–19 and low in 1957–58 (37). Second, fatalities were disproportionately common in young adults in 1918–19. Deaths occurred principally in older age groups in 1957–58 (38).

At first glance, these facts suggest marked differences in the virulence of the viruses of the two pandemics. It appears more probable, however, that factors other than virus virulence, especially the use of antimicrobial drugs, reduced the frequency of severe disease in the recent pandemic. Virtually all well-studied influenza fatalities in 1918–19 were associated with bacterial infections of the lung. Although bacterial pneumonias produced significant mortality in 1957–58, five of 11 or almost 50 per cent of patients in the present study who died had a diffuse hemorrhagic pneumonia apparently produced solely by influenza virus infection. The previously cited studies of other investigators also indicate that influenza virus pneumonia without bacteria was an important cause of death in 1957–58. It is likely that control of many superimposed bacterial pneumonias with drug therapy operated to select such abacterial cases.

The premise that mortality is unrelated to intrinsic virus virulence receives further support from the observation that 80 per cent of the deaths observed in the present series occurred in patients with serious underlying disease. This high coincidence of influenza fatality and underlying disease was not observed in the large number of young adults who died in 1918–19. However, it is probable that the selective crowding of young people under war time conditions together with the lack of effective therapy for the bacterial complications

TABLE XVI

The association of underlying disease with pulmonary complications of influenza: The importance of antecedent cardiac disease

Disease syndrome	No. of patients	Incidence of underlying disease*			Deaths		
		Total no. patients	No. with CV disease	No. with RHD	Total no. who died	No. with underlying disease	No. with RHD who died
Influenza complicated by bacterial pneumonia	15	8†	3	3	1	1	1 of 3
Concomitant influenza virus and bacterial pneumonia	9	7†	5	3	5	3	1 of 3
Primary influenza virus pneumonia	6	6	6	4	5	5	3 of 4
Totals	30	21	14	10	11	9	5 of 10

* CV, cardiovascular disease; RHD, rheumatic heart disease.
† In one of this number the "disease" was pregnancy.

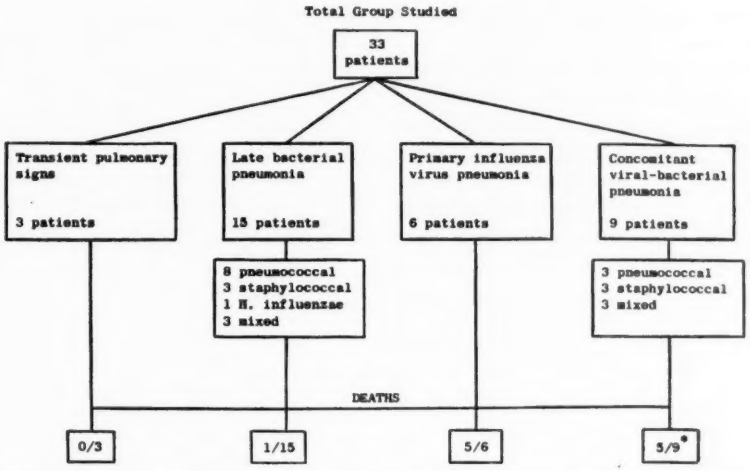

FIG. 30. THE PULMONARY COMPLICATIONS OF INFLUENZA, THE NEW YORK HOSPITAL, 1957–58

Asterisk indicates two late deaths unassociated with acute illness.

of influenza might well account for the high mortality of influenza in young adults at that time. These factors rather than hypothetical changes in the virulence of influenza virus may explain the more terrifying face of pandemic influenza in 1918–19.

It thus emerges that severe and fatal influenza in 1957–58 was usually a disease of previously damaged hosts, as it appears to have been in the interpandemic decades (Table XV). As the problem of bacterial infection, so troublesome in 1918–19, has been modified by antimicrobial drugs, the problem of influenza virus pneumonia in patients with underlying disease has become proportionately more important.

It is to be anticipated that in future influenza epidemics the majority of deaths may well occur in patients with underlying disease who develop influenza virus pneumonia with or without concomitant bacterial infection. In such epidemics the relatively high risk of severe illness and death in the chronically ill may be reduced by prior specific immunization with influenza virus vaccines.

SUMMARY

1. During a prospective study of the 1957–58 influenza epidemic, 33 patients with pulmonary complications of influenza were admitted to The New York Hospital-Cornell Medical Center (see Figure 30). As these patients were studied it became apparent that their disease could be categorized on the basis of clinical and laboratory investigations into four distinct syndromes:

a) In three patients transient pulmonary signs were detected in the absence of roentgenographic evidence of pneumonia. These patients did not have bacterial infection, and recovery ensued on symptomatic treatment alone. This syndrome was believed to represent bronchiolitis related to influenza virus infection.

b) In 15 patients secondary bacterial pneumonia arose relatively late in the course of influenza. The etiology of this focal pneumonia was pneumococcal in eight, staphylococcal in three, mixed in three and related to the influenza bacillus in one. The course of infection and the response to therapy of these patients did not differ materially from that of uncomplicated bacterial pneumonia. Influenza infection was proved serologically, but virus could not be recovered from any of these patients.

c) In nine patients laboratory and clinical study indicated that concomitant infection with influenza virus and bacteria was responsible for the pneumonia observed. Of these patients, three had pneumococcal, three staphylococcal and three mixed bacterial infections. Despite adequate antibacterial therapy, three patients died during their acute illness, one with pneumococcal and two with staphylococcal infections. The Asian type of influ-

enza A virus was isolated from the lungs of these three patients.

d) Six patients developed a fulminating diffuse hemorrhagic pneumonia which led to death in five. No pathogen other than influenza virus could be related to this abacterial syndrome. These patients pursued a relentless course characterized by extreme dyspnea, hypoxia and failure to respond to antibacterial drugs. Influenza virus was isolated from five of these six patients, and directly from the lungs of four who died within 10 days of onset. Study of postmortem cultures and tissue sections of lungs failed to reveal bacteria.

2. Twenty-four of the 33 patients with pulmonary complications of influenza had chronic, underlying disease or were pregnant. Twenty-one of the 30 patients with roentgenographically confirmed pneumonia had pre-existing illness or pregnancy. Fourteen of these 21 had underlying cardiac disease which was of rheumatic origin in 10. Eleven patients died. Nine of the deaths occurred in patients with underlying disease, and five of these patients had rheumatic heart disease.

3. Myocarditis clearly related to influenza was not observed in this series of severely ill patients.

4. Signs suggestive of encephalitis were observed in three patients, but influenza virus was not recovered from the spinal fluid. Pathologic evidence of encephalitis was not found in four brains examined at autopsy.

5. A review of recent and earlier literature on pulmonary complications of influenza supports conclusions derived from the present study that:

a) Influenza virus infection *per se* may induce pneumonia in the absence of other microbial pathogens.

b) Underlying disease, and more particularly rheumatic heart disease, may be of importance in the genesis of influenza virus pneumonia.

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REFERENCES

1. Rogers, D. E., Louria, D. B., and Kilbourne, E. D. The syndrome of fatal influenza virus pneumonia. Trans. Ass. Amer. Phycns Suppl. 1958. In press.
2. Goodpasture, E. W. The significance of certain pulmonary lesions in relation to the etiology of influenza. Amer. J. med. Sci. 1919, **158**, 863.
3. Smith, W., Andrewes, C. H., and Laidlaw, P. P. A virus obtained from influenza patients. Lancet 1933, **2**, 66.
4. Mulder, J., and Masurel, N. Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. Lancet 1958, **1**, 810.
5. Blumenfeld, H. L., Kilbourne, E. D., Louria, D. B., and Rogers, D. E. Studies on influenza in the pandemic of 1957–1958. I. An epidemiologic, clinical and serologic investigation of an intra-hospital epidemic, with a note on vaccination efficacy. J. clin. Invest. 1959, **38**, 199.
6. Podosin, R. L., and Felton, W. L., II. The clinical picture of Far East influenza occurring at the Fourth National Boy Scout Jamboree: Report of 616 cases. New Engl. J. Med. 1958, **258**, 778.
7. Kilbourne, E. D. Studies on influenza in the pandemic of 1957–1958. III. Isolation of influenza A (Asian strain) viruses from influenza patients with pulmonary complications. Details of virus isolation and characterization of isolates, with quantitative comparison of isolation methods. J. clin. Invest. 1959, **38**, 266.
8. Blair, J. E., and Carr, M. The bacteriophage typing of staphylococci. J. infect. Dis. 1953, **93**, 1.
9. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
10. Van Slyke, D. D., and Sendroy, J., Jr. Studies of gas and electrolyte equilibria in blood. XV. Line charts for graphic calculations by Henderson-Hasselbalch equation and for calculating plasma carbon dioxide content from whole blood content. J. biol. Chem. 1928, **79**, 781.
11. Chinard, F. P. Estimation of plasma volume by dye dilution method *in* Methods in Medical Research. Year Book Publishers, Inc., 1951, vol. 4, p. 38.
12. Noble, R. P., and Gregerson, M. I. Blood volume in clinical shock. I. Mixing time and disappearance rate of T-1824 in normal subjects and in patients in shock; determination of plasma volume in man from 10-minute sample. J. clin. Invest. 1946, **25**, 158.
13. Bessman, S. P. Personal communication.
14. Friedemann, T. E., and Haugen, G. E. Pyruvic acid. II. The determination of keto acids in blood and urine. J. biol. Chem. 1943, **147**, 415.
15. Parker, F., Jr., Jolliffe, L. S., Barnes, M. W., and Finland, M. Pathological findings in the lungs of five cases from which influenza virus was isolated. Amer. J. Path. 1946, **22**, 797.
16. Borden, C. W. Acute myocarditis; report of case with observations on etiologic factor. Amer. Heart J. 1950, **39**, 131.

17. Scadding, J. G. Lung changes in influenza. Quart. J. Med. 1937, n.s. **6**, 425.
18. Stuart-Harris, C. H., Andrewes, C. H., and Smith, W. A study of epidemic influenza: With special reference to the 1936–37 epidemic. Medical Research Council, Special Report Series No. 228. London, His Majesty's Stat. Off., 1938.
19. Finland, M., Barnes, M. W., and Samper, B. A. Influenza virus isolations and serological studies made in Boston during the winter of 1943–1944. J. clin. Invest. 1945, **24**, 192.
20. Maxwell, E. S., Ward, T. G., and Van Metre, T. E., Jr. The relation of influenza virus and bacteria in the etiology of pneumonia. J. clin. Invest. 1949, **28**, 307.
21. Giles, C., and Shuttleworth, E. M. Post-mortem findings in 46 influenza deaths. Lancet 1957, **2**, 1224.
22. Hers, J. F. P., Goslings, W. R. O., Masurel, N., and Mulder, J. Deaths from Asiatic influenza in the Netherlands. Lancet 1957, **2**, 1164.
23. Perrier, C. V., Hausser, E., and Paccaud, M. F. Sur quelques cas de broncho-pneumonie grippale d'évolution fatale: isolement du virus A/Asia/57 dans deux cas. Praxis 1957, **46**, 1121.
24. Rock, J. A., Braude, A. I., and Moran, T. J. Asian influenza and mitral stenosis. Report of a case with autopsy. J. Amer. med. Ass. 1958, **166**, 1467.
25. Commission on Acute Respiratory Diseases, Ft. Bragg, N. C., and The New York State Dept. of Health, Albany, N. Y. The relation between epidemics of acute bacterial pneumonia and influenza. Science 1945, **102**, 561.
26. Stuart-Harris, C. H., Laird, J., Tyrrell, D. A., Kelsall, M. H., and Franks, Z. C. The relationship between influenza and pneumonia. J. Hyg. (Lond.) 1949, **47**, 434.
27. MacCallum, W. G. The pathology of the pneumonia in the United States Army camps during the winter of 1917–1918. Monograph No. 10. New York, The Rockefeller Institute for Medical Research, 1919.
28. Wolbach, S. B. Comments on the pathology and bacteriology of fatal influenza cases as observed at Camp Devens, Mass. Bull. Johns Hopk. Hosp. 1919, **30**, 104.
29. Opie, E. L., Blake, F. G., Small, J. C., and Rivers, T. M. Epidemic Respiratory Disease; The Pneumonias and Other Infections of the Respiratory Tract Accompanying Influenza and Measles. St. Louis, C. V. Mosby Co., 1921.
30. Himmelweit, F. Influenza virus B isolated from a fatal case of pneumonia. Lancet 1943, **2**, 793.
31. Michael, M., Jr. Staphylococcus aureus pneumonia with special reference to its occurrence as a complication of influenza. J. Amer. med. Ass. 1942, **118**, 869.
32. Chickering, H. T., and Park, J. H. Staphylococcus aureus pneumonia. J. Amer. med. Ass. 1919, **72**, 617.
33. Rogers, D. E. The current problem of staphylococcal infections. Ann. intern. Med. 1956, **45**, 748.
34. Jordan, E. O. Epidemic Influenza. Chicago, American Medical Association, 1927.
35. Abrahams, A., Hallows, N., and French, H. A further investigation into influenzo-pneumococcal and influenzo-streptococcal septicaemia: Epidemic influenzal "pneumonia" of highly fatal type and its relation to purulent bronchitis. Lancet 1919, **1**, 1.
36. Winternitz, M. G., Wason, I. M., and McNamara, F. P. The Pathology of Influenza. New Haven, Yale University Press, 1920.
37. Dunn, F. L. Pandemic influenza in 1957. Review of international spread of new Asian strain. J. Amer. med. Ass. 1958, **166**, 1140.
38. Langmuir, A. D., Pizzi, M., Trotter, W. Y., and Dunn, F. L. Asian influenza surveillance. Publ. Hlth Rep. (Wash.) 1958, **73**, 114.
39. Stokes, J., Jr., and Wolman, I. J. The probable synergism of human influenza virus and staphylococcus aureus in a rapidly fatal respiratory infection. Int. Clin. 1940, **1**, 115.
40. Burnet, F. M., Stone, J. D., and Anderson, S. G. An epidemic of influenza B in Australia. Lancet 1946, **1**, 807.
41. Mulder, J., and Verdonk, G. J. Studies on the pathogenesis of a case of influenza-A pneumonia of three days' duration. J. Path. Bact. 1949, **61**, 55.
42. Evans, A. D, and Evans, M. Staphylococcal infection of the lower respiratory tract in adults with influenza. Lancet 1956, **1**, 771.
43. Deaths from Asian influenza, 1957. A report by the Public Health Laboratory Service based on records from hospital and public health laboratories. Brit. med. J. 1958, **1**, 915.

STUDIES ON INFLUENZA IN THE PANDEMIC OF 1957–1958. III. ISOLATION OF INFLUENZA A (ASIAN STRAIN) VIRUSES FROM INFLUENZA PATIENTS WITH PULMONARY COMPLICATIONS. DETAILS OF VIRUS ISOLATION AND CHARACTERIZATION OF ISOLATES, WITH QUANTITATIVE COMPARISON OF ISOLATION METHODS *

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Because the preceding paper of this series (1) purports to document the entity of primary influenza virus pneumonia, the circumstances of the recovery of viruses from cases of influenza with pulmonary complications will be presented here in detail. In addition, the present paper will describe a preliminary study of certain properties of viral isolates from fatal and nonfatal cases, and corollary data on viral isolation methods.

MATERIALS AND METHODS

Collection of human materials for viral isolation. Throat washings with sterile 0.01 M phosphate buffered saline were collected by gargling and frozen within one hour of collection in a CO_2-ethanol mixture prior to storage at − 68° C. Sections of *viscera, urine* and *spinal fluid* were similarly frozen and stored. *Blood* collected in acid-citrate-dextrose (ACD) was subjected to low speed centrifugation (500 G) at 2° C.; cellular and plasma fractions were frozen and stored separately. Prior to their inoculation, specimens were rapidly thawed in running cold tap water; streptomycin in a final concentration of 1.0 mg. per ml. and penicillin in a final concentration of 1,000 units per ml. were added. Throat washings were homogenized by refluxing them through a No. 20 needle. *Lung specimens* were weighed (except in the case of small needle aspirates) and homogenized as 5 or 10 per cent antibiotic-saline suspensions (by weight) at 4° C. in either a Lourdes omnimixer or a Ten Broek grinder. Suspensions were centrifuged in plastic tubes at 8,200 G for five minutes at room temperature. Supernatant fluid was used for inoculation and promptly refrozen.

Inoculation procedures. Ten to 11 day old *chick embryos* were used in all experiments unless it is otherwise indicated. Eighteen Gm. CFW male *mice* were employed in mouse lung studies. *Monkey kidney cells* for tissue cultures were purchased from Microbiological Associates, Inc. in flasks. Tubes were seeded with 50,000 cells per tube. Cultures were grown for 48 hours before inoculation with a medium developed in this laboratory which contains 5 per cent calf serum and 0.5 per cent lactalbumin hydrolysate in Earle's balanced salt solution (pH 7.0). Before inoculation, cells were washed three times with 2 ml. per tube of phosphate buffered 0.85 per cent saline; medium 199 was added as the maintenance medium. Incubation was effected at 35° C. Three days after inoculation fluids were changed to forestall toxic effects of inocula (2), and tested for influenza virus hemagglutinin. After additional incubation for four days, fluids were again tested for hemagglutinin, pooled and subjected to additional passage for nine days. Tubes were observed daily for cytopathogenic effects.

Hemagglutinin titrations were performed by the addition of human O red blood cells (RBC) in initial concentration of 0.5 per cent to serially diluted viral suspensions. End points were determined after 60 minutes at 22° C. by direct reading of tubes for hemagglutination patterns. Infectivity titrations of virus involved departures from usual methods and are described under "Results."

Electron microscopy was done by Dr. James Murphy of The Rockefeller Institute for Medical Research by a technique described earlier by Murphy and Bang (3).

RESULTS

A comparison of methods of viral isolation

Early in the present study it became evident that influenza virus hemagglutinin was frequently noted in allantoic fluids from eggs inoculated by the amniotic route in primary passage isolation attempts. This observation, which suggested an unusual primary ability of the 1957 viruses to multiply in the allantoic sac without initial amniotic passage, prompted a quantitative comparison of the sensitivity of the amniotic and allantoic sacs to infection with small amounts of virus from human specimens. Although a frequently-quoted

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TABLE I

Simultaneous titrations by amniotic and allantoic routes of influenza virus of human lung suspension (Case G. H.)

Lung suspension dilution	Eggs positive for hemagglutinin when injected by: Amniotic route*	Allantoic route*
10^{-2}	**+ + +** D D D	**+ + + +**
10^{-3}	**+ + + + + +**	**+ + + +**
10^{-4}	**0 0 0 0 0** +	**0 +** + +
10^{-5}	**0 0 0 0 0 0**	**0 0 0 0**
10^{-6}	**0 0 0 0 +** D	**0 0 0 0**
1st passage titer	$10^{-2.7}$	$10^{-3.5}$
2nd passage (cumulative) titer with subinoculations	$10^{-3.8}$	$10^{-4.3}$

* Bold face **+** and **0** indicate results on 1st passage; + indicates 1:4 dilution of amniotic or allantoic fluid positive for hemagglutinin; 0 indicates 1:4 dilution negative; D indicates egg dead within 48 hours after injection, fluid not tested.

earlier paper (4) had indicated a much greater sensitivity of the amniotic route (in 13 day chick embryos) compared with the allantoic (in 11 day embryos), use of 13 day embryos has proved cumbersome in the hands of the present investigator. Amniotic fluid harvests are small in volume; fluids are opaque and high in urates and protein and often induce nonspecific hemagglutination in low dilution. The use of younger embryos of 9 to 11 days circumvents these difficulties and has proved successful in the past (5). Accordingly, comparison of amniotic and allantoic routes has been made in younger embryos of identical age and with the employment of identical incubation periods. In Table I are presented the results of simultaneous titration of a suspension of human lung by amniotic and allantoic injection of separate groups of 10 day old chick embryos. Amniotic inoculations were performed with a 1.5 inch No. 25 needle by the "blind" technique (6) under transillumination with a Prevue® egg candler; allantoic inoculation was effected by injection directly into the allantoic sac through a hole drilled in the side of the egg, also with a No. 25 needle. One-tenth ml. volumes were injected by either route. Eggs were incubated at 35° C. for 60 to 65 hours prior to harvest of individual fluids for hemagglutinin. The presence of hemagglutinin at the 10^{-6} dilution in one egg inoculated amniotically suggested the possibility that virus below the concentration required for hemagglutination might be present in the "skipped" dilutions which were negative for hemagglutinin. Each negative fluid (*not* in bold face in the table) was injected undiluted by the allantoic route into two eggs which were incubated for a further 40 hours. This subinoculation did in fact reveal latently positive eggs in the initial titration but did not contribute appreciably either to elevation of apparent viral titers or to the relative concentrations of virus revealed by the two routes of inoculation. By either endpoint the allantoic method appeared certainly no less sensitive than the amniotic to high dilutions of virus.

The possibility was considered that this result might be characteristic only of virus recovered directly from human tissue or from fatal cases of influenza. However, titration under similar conditions of virus in the throat washing of a nonfatal case (Table II) again attested to the comparable sensitivity of allantoic and amniotic routes of inoculation.

Further isolations by the allantoic route—The influence of cortisone

In view of the possibility that these viruses from only two cases may have been atypical in behavior, four additional throat washings which had previously yielded virus upon amniotic inoculation were injected (undiluted) by the allantoic route into groups of six 10 day old eggs which were subsequently incubated for 60 hours. Results of first and second passages by this method are presented in Table III. Undiluted allantoic pools were em-

TABLE II

*Simultaneous titrations of a throat washing by amniotic and allantoic routes (Case R. M.)**

Throat washing dilution	Eggs positive for hemagglutinin when injected by: Amniotic route†	Allantoic route†
10^{0}	+ + D D	+ + + +
10^{-1}	+ + + + + +	+ + + +
10^{-2}	+ + 0 0 D	+ + 0 0
10^{-3}	0 0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0 D	0 0 0 0

* Nonfatal case of influenza.

† +, 1:4 dilution of amniotic or allantoic fluid positive for hemagglutinin; 0, 1:4 dilution negative; D, egg dead within 48 hours after injection, fluid not tested.

TABLE III

Recovery of influenza viruses by allantoic inoculation from throat washings previously positive by amniotic inoculation

Case	1st Allantoic passage			2nd Allantoic passage		
	Eggs injected with:	No. eggs positive for hemagglutinin*	Geometric mean titer†	Eggs injected with:	No. eggs positive for hemagglutinin*	Geometric mean titer†
Z. C.	H_2O	+ + + 0 0 0	2.8	H_2O	+ + + + +	6.0
	Cortisone	+ 0 0 0 0 0	1.2	Cortisone	+ + + + +	7.0
				H_2O	+ + + + +	7.0
				Cortisone	+ + + + +	7.2
R. S.	H_2O	0 0 0 0 0 0	<1.0			
	Cortisone	0 0 0 0 0 0	<1.0	H_2O	+ 0 0 0 0	1.4
				Cortisone	+ 0 0 0 0	1.4
				H_2O	+ + + + +	5.4
S. D.	H_2O	+ 0 0 0 0 0	1.4	Cortisone	+ + + + +	6.2
	Cortisone	+ 0 0 0 0 0	2.0			
				H_2O	+ + + + +	6.8
				Cortisone	+ + + + +	8.4
A. B.	H_2O	+ + + + + +	5.6	H_2O	+ + + + +	6.2
	Cortisone	+ + + + + +	7.1	Cortisone	+ + + + +	7.4

* At 1:4 dilution of allantoic fluid.
† Log_2 viral hemagglutinin (*e.g.*, 6.0 = conventional titer of 1:64).

ployed for second passage inocula. Either hydrocortisone diethylaminoacetate hydrochloride ("cortisone"), 0.001 mg., or distilled H_2O was injected two hours prior to virus-containing materials. It was expected that cortisone, by inhibiting the possible interfering effects of inactive or nonreplicating virus of the inocula (7), might expedite demonstration of virus by enhancing final virus yields. It may be noted in Table III that cortisone was essentially of no practical value in effecting viral isolations although a trend toward higher concentrations of hemagglutinating virus was usually observed with the steroid.

In comparison with previous amniotic isolations, three of the four washings yielded hemagglutinin on initial allantoic passage. The sole exception, the throat washing of Case R.S., yielded a peculiarly slow-growing virus which is still under investigation. Repetition of the second allantoic passage with this virus with 10^0 and 10^{-2} dilutions of inocula resulted in detectable hemagglutinin only with the undiluted inoculum even after 72 hours of incubation. The data summarized in Table III support the evidence presented earlier concerning the effectiveness of the allantoic method of inoculation.

Other methods of viral isolation

The reported use of monkey kidney tissue culture for influenza virus isolation (8, 9) prompted a comparison of this method of viral propagation with chick embryo techniques. In addition, the possibility that isolates from human lung might multiply best in pulmonary tissue suggested a trial of mouse inoculation despite the reported insensitivity of this host in primary isolation attempts (10). Groups of mouse lungs (removed three days after intranasal inoculation of mice with varying dilutions) were ground as 5 per cent suspensions in saline with antibiotics in a blender, then subjected to centrifugation at room temperature of 8,200 G for five minutes. Supernatant fluids were subinoculated by the allantoic route into 10 day old eggs which were then incubated for 65 hours; allantoic fluids were tested at 1:4 dilution for viral hemagglutinin. The result of a titration by this method of virus in a human lung may be seen in Table IV. It will be noted that combined mouse-egg passage (11) yielded virus only at the initial dilution of 10^{-1} while single passage allantoic inoculation of 10 day old eggs detected

TABLE IV

Simultaneous titrations by chick embryo (allantoic), mouse-chick embryo, and tissue culture methods of influenza virus in human lung (Case C. P.)

	Chick embryo (allantoic)	Mouse-chick embryo	Monkey kidney tissue culture
Titer ID_{50}	$10^{-4.7}$	10^{-1}	$<10^{-1}$

TABLE V

Attempted isolation of influenza virus by mouse inoculation of throat washings previously tested in eggs*

Case	Throat washing tested in eggs was:			Result of isolation attempt in mice:	
	Positive	Estimated viral concentration†	Negative	Positive	Negative
Z. C.	+	+			+
R. S.	+	+			+
S. D.	+	+			+
A. B.	+	+++		+	
M. E.			+		+
A. M.			+		+
D. P.			+		+
B. N.			+		+
M. M.			+		+

* Groups of five mice were inoculated intranasally with the throat washing; lungs were harvested two days later, pooled and homogenized as 5 per cent suspensions by weight. Groups of five eggs were inoculated with the 5 per cent lung homogenates; allantoic fluids harvested 60 hours later were tested for hemagglutinin.

† + indicates no allantoic fluids positive for viral hemagglutination on first passage. +++ indicates one-half of allantoic fluids positive for viral hemagglutination on first passage or actual titration of EID_{50} per 1.0 Gm. of lung (wet weight) by chick embryo allantoic route.

virus at a dilution of more than 10^{-4}. No dilution inoculated into monkey kidney tissue cultures induced demonstrable cytopathogenic effects or hemagglutinin formation despite subinoculation as outlined in "Materials and Methods." Inoculation of monkey kidney tissue cultures with the four throat washings positive by both amniotic and allantoic injection (Table III) similarly failed to demonstrate virus.

A similar test of the efficiency of the mouse-egg method resulted in the recovery of virus from only one of four throat washings previously shown to contain virus by primary egg inoculation (Table V). This sole positive specimen had had the highest estimated virus concentration when inoculated into eggs. No throat washings negative in eggs were positive with any other method.

Recovery of virus from patients with pulmonary complications

By the use of the amniotic or allantoic inoculation techniques which have been described, hemagglutinating viruses were recovered from specimens of lungs or throat washings of 14 influenza patients in whom acute pulmonary signs were apparent. A record of these viral isolations is listed in Table VI.

In two patients (G.S. and C. B.) only transient signs of pulmonary infection were evident and bacterial infection was not demonstrated (1). As discussed in the preceding paper of this series (1), six patients showed no evidence of bacterial pneumonia despite signs of extensive pulmonary consolidation. From five of these patients who died (Cases G.H., C.P., A.B., J.B. and A.C.) virus was recovered with ease from either lung suspensions or throat washings. Further, virus was usually present in high concentration as estimated by the frequency of hemagglutinin-positive allantoic fluids following initial amniotic inoculation (see footnote to Table VI). (Amniotic fluids were often pooled during the harvest so that individual hemagglutinin determinations on these fluids were not always feasible.)

The ease with which virus was demonstrated in the lungs of fatal cases was related directly to its subsequently determined concentration (Cases G.H., C.P., A.B., F.R., A.Z.), and was (with the exception of Case A.C.) inversely related to the estimated duration of illness prior to death. In the case of Patients F.R. and A.Z., in whom pulmonary virus was demonstrated with difficulty, lung sections were excised at autopsy nine to 10 days after the onset of illness. In light of the fact that circulating antibody may be demonstrated as early as the seventh day of influenza (12), it is understandable that virus was not demonstrable in high concentration in these cases. Indeed, influenza virus is detected with difficulty in the lung of the mouse nine days after initiation of *infection* (13). Virus was reisolated (in the course of titrations) from the four of the six fatal cases in which pulmonary virus was previously demonstrated in concentrations greater than 10^2 EID_{50} per Gm. of lung. In Case A.B. a small needle aspirate was obtained just ante mortem. This lung fragment was not weighed, so that the titration figure of 10^4 (Table VI) is probably below the actual concentration of virus, as the tissue was diluted an undetermined amount in homogenization of the specimen. In all instances when death occurred before the tenth day of illness virus was recovered from the lung.

Cases of influenza with pulmonary complications from which virus was not isolated

From 15 patients with clinical influenza and evidence of pulmonary disease, influenza viruses

TABLE VI

Recovery of influenza viruses from patients with pulmonary complications

Case	Bacterial complications	Specimen source*	Day specimen obtained†	Virus recovered	Estimated virus concentration‡	Virus reisolated
G. S.§	None	T.W.	2	+	++	N.D.**
C. B.§	None	T.W.	5	+	++	N.D.
		RBC	5	0		
G. H.	None	Lung	5	+	+++ ($10^{5.3}$)	2x
C. P.	None	Lung	5	+	+++ ($10^{5.7}$)	2x
A. B.	None	Lung‖	4	+	+++ (10^{4})	1x
		T.W.	3	+	+++	2x
		Urine	3	0		
J. B.	None	T.W.	3	+	+++	N.D.
		Lung	10	0		
		CSF	5	0		
		Urine	3	0		
A. C.	None	T.W.	3	+	+++	N.D.
		Lung	4	+	+ ($<10^{2}$)	
P. E.	Pneumococcal	T.W.	3	+	+	N.D.
		CSF	6	0		
E. R.	Pneumococcal	T.W.	1	+	+	N.D.
M. L.	Pneumococcal	T.W.	3	+	+++	N.D.
		RBC	3	0		
F. R.	Pneumococcal	T.W.	? 1–4	+	+++	N.D.
		Lung	? 6–10	+	+ ($<10^{2}$)	0
		RBC	? 1–4	0		
G. A.	Staphylococcal	T.W.	3	+	+++	N.D.
A. Z.	Staphylococcal	T.W.	4	+	+	N.D.
		Lung	10	+	+ (10^{2})	0
		Urine	4	0		
		RBC	4	0		
B. P.	Staphylococcal	Sputum	5	0		
		Lung	5	+	+++ ($10^{2.7}$)	1x

* T.W., throat washing; RBC, red blood cells; CSF, cerebrospinal fluid.

† Day of illness.

‡ + indicates no allantoic fluids positive for viral hemagglutinin on 1st passage. ++ indicates < one-half of allantoic fluids positive for viral hemagglutinin on 1st passage. +++ indicates one-half of allantoic fluids positive for viral hemagglutinin on 1st passage or actual titration of EID_{50} per 1.0 Gm. of lung (wet weight) by chick embryo allantoic route.

§ Transient pulmonary signs noted.

‖ Lung puncture.

** N.D., reisolation not attempted.

were not isolated despite serological substantiation of infection in all cases (1). However, consideration of Table VII shows that in only four of these cases (*27* per cent) were throat washings obtained earlier than the fifth day of illness. It is uncommon to recover virus after this time (14). In most of these patients a secondary bacterial complication had ensued—further evidence that viral isolation was essayed relatively late in the course of the primary influenza virus infection (1).

Failure to demonstrate virus in nonrespiratory sources

Demonstration that a viremia may occur in mice infected with influenza virus (15) led to reinvestigation of the possibility of viremia in human disease. To this purpose specimens of erythrocytes and urine were studied which were obtained early in illness from patients in whom respiratory tract virus had been demonstrated. Despite concentration of urine 3- to 10-fold and the expectation that blood stream virus might have been adsorbed *in vivo* to RBC suspensions, attempts at virus recovery were fruitless. Spinal fluids from patients who exhibited neurological symptoms were also negative for virus.

Identification of viral isolates

All isolates from either lungs or throat washings were serologically identified as Asian strain influenza A viruses by hemagglutination-inhibition tests. Complex antigenic analysis of isolates by

TABLE VII

Serologically proved cases of influenza with pulmonary complications from which virus was not recovered

Case	Bacterial complications	Specimen source	Day of illness on which specimen was obtained
G. V.*	None	T.W.†	2
D. P.	Pneumococcal	T.W.	7
H.G.	Staphylococcal	T.W.	7
		Urine	8
P. B.	None	T.W.	2
B. N.	Aerobacter	T.W.	15
M. M.	Pneumococcal	T.W.	6
P. R.	Pneumococcal	T.W.	4
		Urine	5
C. G.	*H. influenzae* and staph.	T.W.	12
R. L.	Pneumococcal	T.W.	7
N. M.	Pneumococcal	T.W.	6
R. C.	Staphylococcal	T.W.	5
		Lung	53
A. L.	Pneumococcal	T.W.	1
H. W.	Staphylococcal	T.W.	35
L. F.	Staphylococcal	T.W.	9
A. M.	Pneumococcal	T.W.	7

* Rales on auscultation; no roentgenographic evidence of infiltrate.
† T.W., throat washing.

the production of isolate-specific antisera was not done. However, all isolates were inhibited to at least the same degree as the homologous virus by antiserum for the Japan 305 strain of influenza A virus.

A comparison of viral isolates from lungs and throat washings of fatal and nonfatal cases

It has been stressed in the foregoing paper (1) that fatal influenza occurred usually in a setting of abnormal host physiology. There is, therefore, no need to postulate an increased virulence of the virus infecting and recovered from such cases. There is, in fact, no biological characteristic of influenza viruses which has been correlated with their virulence for man, and thus no way of demonstrating that one viral isolate differs in virulence from another. The problem is complicated further by the probable heterogeneity of viral particles in any given isolate, and the inevitable shifting of the proportion of each type of particle with laboratory cultivation of the virus. It is therefore difficult to relate the sum of the properties of even early passage egg-adapted virus to the pristine character of this virus as it induced infection in man. Nevertheless, crude correlations may be drawn in that it can be stated that the somewhat greater capacity of influenza A viruses to induce more severe illness in man than is caused by influenza B viruses parallels the relatively greater capacity of influenza A viruses to damage the lung of the mouse in early passages.

Although an earlier experiment (Table V) had failed to show that viruses in human lung suspensions (from fatal cases) were unduly capable of initiating murine infection, it was considered of interest to compare the relative pulmonary toxicity of the larger amounts of such viruses provided by cultivation in eggs. As outlined in Table VIII, equivalent masses of virus derived from either the throat washings of nonfatal cases or the lungs of fatal cases were inoculated intranasally into groups of 10 mice each. Viruses were at the stage of either second or third egg passage. Mice were killed after five days and gross pulmonary lesions were scored (16). Only with virus from a nonfatal case were significant lesions produced.

TABLE VIII

Mouse lung toxicity of influenza viruses isolated in eggs from fatal and nonfatal cases of influenza

Case	Fatal	Source of isolate	Egg passage of isolate*	Viral inoculum		Total lesion score (10 mice)
				Original HA titer†	Dilution	
R. S.		T.W.	Am_1 All_1	2,048	1:16	0
R. M.		T.W.	All_2	128	0	0
S. D.‡		T.W.	Am_1 All_1	512	1:4	24
C. P.	Died	Lung	All_2	128	0	1
A. B.	Died	Lung	All_2	256	1:2	3
A. Z.	Died	Lung	Am_1 All_2	128	0	0

* Am, amniotic route; All, allantoic route.
† Reciprocal of the maximal dilution of the preparation which induces hemagglutination.
‡ Original throat washing negative in mice (Table V).

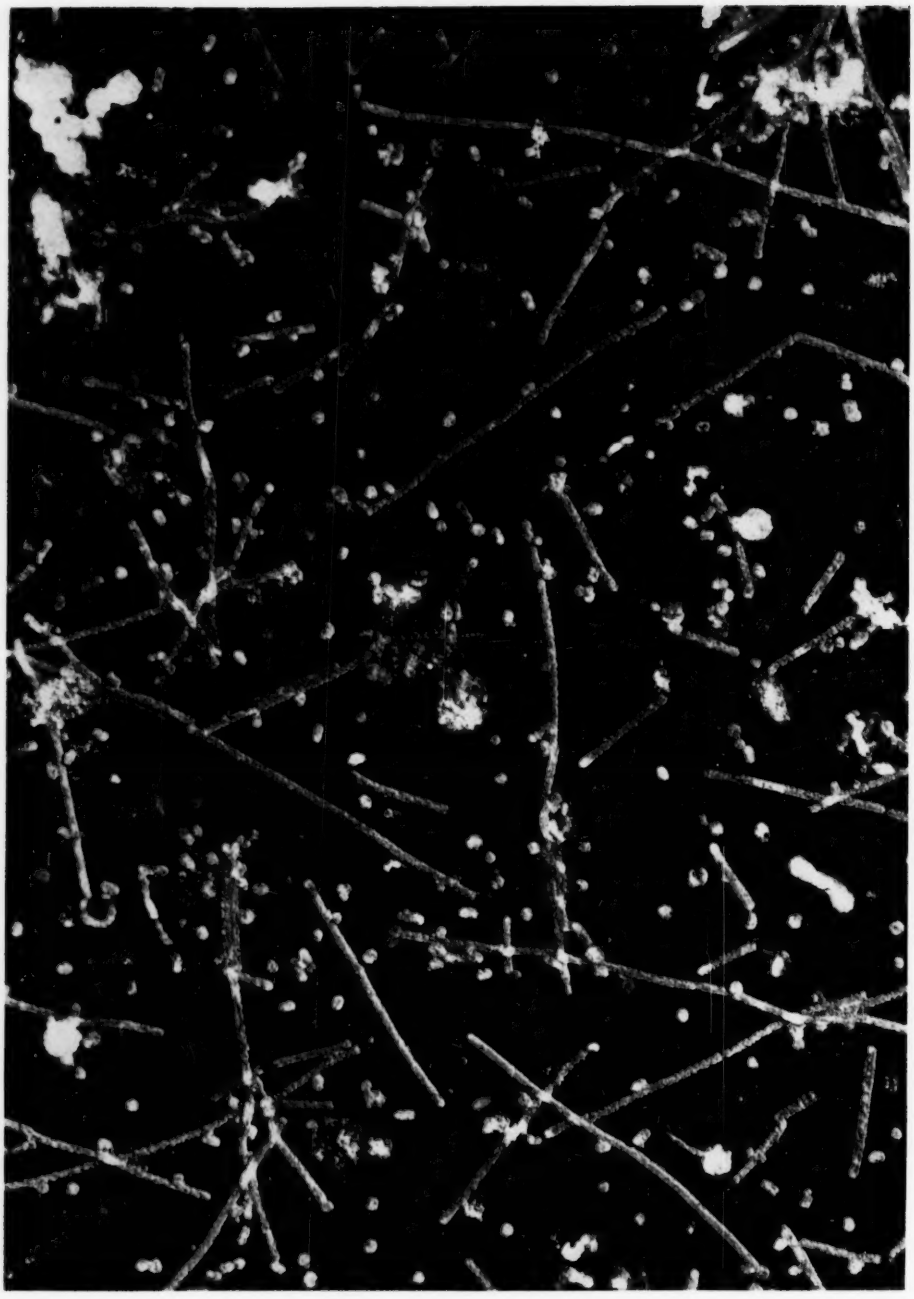

FIG. 1. ELECTRON MICROSCOPY (× 19,600) OF INFLUENZA VIRUS ISOLATE FROM THE THROAT WASHING OF A NONFATAL CASE (CASE R.M.)

Chromium shadow—OsO_4 fixation. Both filamentous and spheroidal forms of the virus are illustrated. This picture is representative of all isolates which were examined from fatal and nonfatal cases. Electron microscopy by Dr. James S. Murphy.

Therefore, further investigations along this line did not appear promising.

The morphology of a number of isolates was examined by electron microscopy. In three experiments performed in collaboration with Dr. James Murphy of The Rockefeller Institute for Medical Research, isolates from three nonfatal cases without pulmonary involvement and from lung and throat washings of six fatal cases were examined by electron microscopy. Viral preparations were made by a technique which has been previously reported (3). Although detailed examination (with actual measurements of particles) was not undertaken, it was apparent that marked differences among isolates were not readily discernible. In most preparations filaments predominated. A preparation representative of the morphology of both "fatal" and "nonfatal" isolates is illustrated in Figure 1. Spherical and filamentous forms of the virus may be observed in this photograph.

Hemagglutinating characteristics of viruses from fatal and nonfatal cases were studied. In summary, all isolates agglutinated human, sheep and chicken erythrocytes. The relative titers of viral hemagglutination with the various cells did not differ significantly regardless of the origin of the viral isolate.

DISCUSSION

Influenza A virus has been isolated and reisolated from the lungs of influenza patients with nonbacterial pulmonary involvement. In patients who died early in illness the concentration of pulmonary virus was usually high—comparable to the concentrations observed in the experimental disease of mice at a comparable stage of infection. Lower concentrations of virus have been demonstrated in two patients in whom bacterial complications precipitated hospitalization and who were (on the basis of historical evidence) at a later stage of influenza.

An unexpected corollary of the present study has been a demonstration of the equal efficacy of the allantoic and amniotic routes of chick embryo inoculation for recovery of influenza A virus of the Asian strain.

An expected corollary of the study has been the failure to demonstrate differences in viral isolates from fatal and nonfatal cases.

SUMMARY

1. Influenza A virus (Asian strain) was isolated from 14 patients with influenza who exhibited pulmonary complications. From four fatal cases it was recovered directly from lung tissue to the exclusion of any other pathogen. From three fatal cases virus was recovered from the lung coincident with the recovery of pneumococci or staphylococci.

2. With the viruses studied, the chick embryo proved the most susceptible host and the allantoic route of inoculation of this host equaled the amniotic in sensitivity.

3. Limited studies failed to demonstrate differences in toxicity, hemagglutination capacity or morphology of isolates from fatal and nonfatal cases.

REFERENCES

1. Louria, D. B., Blumenfeld, H. L., Ellis, J. T., Kilbourne, E. D., and Rogers, D. E. Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. J. clin. Invest. 1959, **38**, 213.
2. Mogabgab, W. Personal communication.
3. Murphy, J. S., and Bang, F. B. Observations with the electron microscope on cells of the chick chorioallantoic membrane infected with influenza virus. J. exp. Med. 1952, **95**, 259.
4. Hirst, G. K. Direct isolation of influenza virus in chick embryos. Proc. Soc. exp. Biol. (N. Y.) 1945, **58**, 155.
5. Kilbourne, E. D., Anderson, H. C., and Horsfall, F. L., Jr. Concurrent infection with influenza A and B viruses in a single epidemic of influenza. J. Immunol. 1951, **67**, 547.
6. Hirst, G. K. Direct isolation of human influenza virus in chick embryos. J. Immunol. 1942, **45**, 293.
7. Kilbourne, E. D. The influence of cortisone on experimental viral infection. IV. Negation of interference as the mechanism by which cortisone induces increased virus yields. J. exp. Med. 1957, **106**, 863.
8. Takemoto, K. K., Lynt, R. K., Rowe, W. P., Huebner, R. J., Bell, J. A., Mellin, G. W., and Davis, D. J. Primary isolation of influenza A, B and C viruses in monkey kidney tissue cultures. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 308.
9. Vogel, J., and Shelokov, A. Adsorption-hemagglutination test for influenza virus in monkey kidney tissue culture. Science 1957, **126**, 358.

10. Francis, T., Jr., and Magill, T. P. Direct transmission of human influenza virus to mice. Proc. Soc. exp. Biol. (N. Y.) 1937, **36**, 132.
11. Kilbourne, E. D., and Horsfall, F. L., Jr. Mouse-egg neutralization. Neutralization in the mouse of influenza viruses not adapted to the mouse. J. Immunol. 1951, **67**, 431.
12. Horsfall, F. L., Jr., Hahn, R. G., and Rickard, E. R. Four recent influenza epidemics: An experimental study. J. clin. Invest. 1940, **19**, 379.
13. Kilbourne, E. D. Unpublished data.
14. Hirst, G. K. Laboratory diagnostic procedures for influenza *in* Diagnosis of Viral and Rickettsial Infections, F. L. Horsfall, Jr., Ed. New York, Columbia University Press, 1949, p. 5.
15. Hamre, D., Appel, J., and Loosli, C. G. Viremia in mice with pulmonary influenza A virus infections. J. Lab. clin. Med. 1956, **47**, 182.
16. Horsfall, F. L., Jr. Neutralization of epidemic influenza virus. The linear relationship between the quantity of serum and the quantity of virus neutralized. J. exp. Med. 1939, **70**, 209.